The Role of Neutralizing Antibodies in HIV Infection

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1. Zusammenfassung

Neutralisierende Antikörper verleihen einen effizienten Schutz vor vielen Viruserkrankungen und stellen auch bei der Erforschung der Immunantwort gegen das Humane Immunodefizienz Virus (HIV) einen zentralen Forschungsschwerpunkt dar. Neutralisierende Antikörper binden Epitope auf dem viralen Hüllprotein und verhindern den Eintritt des Virus in die Zelle, indem sie die Interaktion zwischen Virus und zellulärem Rezeptor oder die folgende Fusion mit der Wirtszelle hemmen.

In den letzten Jahren wurde ausführlich dokumentiert, dass sich HIV -wie viele andere Viren- in vitro sehr effizient durch Zell-Zell-Kontakte verbreitet. Die Verbreitung von einer Zelle zur Nachbarszelle erfolgt durch Bildung einer sogenannten virologischen Synapse – einer organisierten Kontaktfläche, die sich zwischen infizierten und nicht infizierten Zellen bildet. Dort konzentrieren sich Viruspartikel und zelluläre Rezeptoren, die am viralen Eintrittsprozess beteiligt sind. Ob neutralisierende Antikörper diese effiziente Art der viralen Verbreitung erfolgreich unterbinden können, konnte bis jetzt noch nicht erwiesen werden. In Anbetracht der postulierten Tragweite dieser effizienten Form der Zell-zu-Zell Übertragung, könnte die Fähigkeit neutralisierender Antikörper in virologischen Synapsen zu wirken, deren in vivo Wirksamkeit definieren.

Im Rahmen dieser Arbeit wurden mehrere Aspekte der Verbreitung durch Zell-Zell-Kontakte erforscht und die Wirkung der bereits existierenden neutralisierenden Antikörper und Eintrittsinhibitoren auf diese Verbreitungsart untersucht. In einem ersten Teil entwickelten wir zwei neuartige Methoden, die es uns erlaubten, beide Arten der viralen Verbreitung getrennt voneinander zu untersuchen.

In einem zweiten Teil führten wir eine umfassende Analyse der Wirksamkeit von neutralisierenden Antikörpern und Eintrittsinhibitoren während der Zell-zu-Zell Übertragung durch. Wir stellten fest, dass die Aktivität von neutralisierenden Antikörpern und Inhibitoren bei der Zell-zu-Zell Übertragung von ihrer Wirkungsweise abhängt. Eine wichtige Klasse von neutralisierenden Antikörpern, die die CD4-Bindungsstelle auf dem Oberflächenprotein erkennt, blockiert sehr effizient die Bindung des Virus an seinen primären Rezeptor auf Zielzellen, das CD4-Molekül. Solche Antikörper werden während der natürlichen Infektion produziert und sind in vitro hochwirksam gegen verschiedene Virus-Isolate. Noch ist unklar, warum HIV in Anwesenheit von solch potenten Antikörpern dennoch replizieren kann. Hier zeigen wir, dass diese Antikörper bevorzugt die Verbreitung freier Viren verhindern, wohingegen die Zell-zu-Zell Übertragung kaum beeinflusst wird.

Noch sind weitere Versuche notwendig, um den relativen Beitrag der Zell-assoziierten und der zellfreien Infektion bestimmen zu können. Eine detaillierte Charakterisierung und Quantifizierung der Zell-zu-Zell-Übertragung ist für die Untersuchung der erforderlichen Immunantwort und für die zukünftige Entwicklung von Impfstoffen von zentraler Bedeutung.

2. Research summary

Neutralizing antibody responses confer protection in a number of viral diseases and have hence been a focus in the investigation of the humoral immune response against the human immunodeficiency virus (HIV). Neutralizing activity is attributed to antibodies directed against specific epitopes on the envelope glycoproteins. They are considered to limit viral entry, by blocking virion attachment to its receptors or by inhibiting membrane fusion.

Over recent years is has been well documented that HIV like many other viruses spreads in vitro very efficiently, if not preferentially, by cell-cell contacts. Viral transmission occurs by formation of a so-called virological synapse – an organized contact area that forms between infected and non-infected cells where virus particles and cellular receptors involved in the entry process are concentrated. Whether neutralizing antibodies are capable of blocking viral transmission between cells remains unclear. Considering the suggested importance of cell-cell transmission the capacity of neutralizing antibodies to act within virological synapses may largely determine their in vivo efficacy.

In this thesis several parameters of HIV cell-cell transmission were studied in the context of existing neutralizing antibodies and entry inhibitors. In a first part, the development of laboratory assays for the assessment of cell-cell transmission was explored. We developed two novel sensitive cell-cell transmission assays based on different approaches, which enable the analysis of this efficient mode of transmission separated from cell-free infection.

In a second part, we performed an extensive analysis of potency of neutralizing antibodies and entry inhibitors in blocking cell-cell transmission. We found that the activity of neutralizing antibodies and inhibitors during cell-cell transmission varies depending on their mode of action. An important class of neutralizing antibodies directed to the CD4 binding site on the virus envelope very efficiently block binding of the virus to its primary receptor on target cells, the CD4 molecule. This type of antibodies is elicited in patients during natural infection and once isolated from infected individuals has shown to be highly potent against different virus isolates. Why HIV still replicates in the presence of such potent neutralizing antibodies remains unclear. Here we show that CD4 binding site antibodies are dramatically less potent inhibitors of cell-cell transmission, and therefore act preferentially by blocking free virus transmission while allowing HIV to spread through cell-cell contact.

Further experiments are necessary to determine the relative contribution of cell free and cell associated infection in vivo. However as the results of my thesis show, it is clear that a detailed characterization and quantification of the relative contribution of cell-cell transmission will be of central importance for the definition of relevant immune responses and future vaccine design.

3. Background

3.1. HIV epidemic

At the beginning of the fourth decade of the HIV epidemic, spread of HIV has finally halted and incidence rates have begun to decrease. As a result of the success of the antiretroviral therapy (ART), the number of new infections has been reduced by approximately 20% since 1999, the year in which the epidemic peaked globally. In 2009 alone, the number of people receiving antiretroviral therapy, increased 30% in a single year (UNAIDS 2010).

Even though this breakthrough is mirrored in decreasing death rates among the estimated 33 million people living with HIV to date, the fact that only a third (5.2 millions) of the estimated 15 million HIV positive persons in low-and middle-income countries in need of treatment, have access to the antiretroviral therapy, reveals that the progress is real but still very fragile (Schwartlander, Grubb et al. 2006; Boyd and Cooper 2007).

The limited access to ART has given impetus to support prophylactic measures such as condom use (Weller and Davis 2002), male circumcision (Siegfried, Muller et al. 2009) and microbicides, topical applied antiretrovirals (Abdool Karim, Abdool Karim et al. 2010), to prevent HIV infection. While condom use as well as male circumcision can reduce transmission rates notably (Auvert, Taljaard et al. 2005; Bailey, Moses et al. 2007; Gray, Kigozi et al. 2007), they have not been supplied sufficiently to stop the epidemic. A recent proof-of concept study examining the effect of a microbicidal vaginal gel, reported a reduction of HIV infection by estimated 39% (Abdool Karim, Abdool Karim et al. 2010). However, the unexpected finding of a wider sub-Saharan African study that this microbicidal gel, when prescribed daily, is not effective in preventing HIV infection, has led to the suspension of this follow up trial (MTN 2011). Therefore, despite the unprecedented efforts to prevent sexually transmitted HIV infection (Grosskurth, Mosha et al. 1995; Weller and Davis 2002; Gray, Kigozi et al. 2007), the measures developed so far will most likely not suffice to halt the epidemic.

A vaccine will be probably required to ultimately eliminate AIDS. But, in contrast to the success of antiretroviral treatment which enables patients to control viremia for many years (Zolopa 2010), HIV vaccine trials in humans have resulted in either no or relatively low protection despite measurable immunogenicity of administered HIV antigens (Flynn, Forthal et al. 2005; Gilbert, Peterson et al. 2005; Buchbinder, Mehrotra et al. 2008; McElrath, De Rosa et al. 2008; Rerks-Ngarm, Pitisuttithum et al. 2009; Gray, Allen et al. 2011). The first vaccine trial to show potential protection was the RV144 Thai vaccine trial, which reported a partial efficacy with a 31% lower infection in vaccinees than in those individuals receiving placebo (Rerks-Ngarm, Pitisuttithum et al. 2009). The vaccine trial generated robust antibody responses targeting the HIV envelope protein but did

not stimulate T cell responses, encouraging new hope that engaging the humoral response to achieve a protective vaccine against HIV may be attainable. There is now a major research focus on aiming to understand the unique aspect of these generated antibodies and the underlying mechanisms leading to protection.

3.2. HIV-1 Genome Organization and Virion Structure

The HIV genome encodes three structural and enzymatic polyproteins - common to all retroviruses- env (envelope), gag (group-specific antigen) and pol (polymerase), and six auxiliary proteins tat, rev, nef, vif, vpr and vpu (Freed 2001) (Figure 1 Organization of the HIV genome). Tat (transactivator) and rev (regulator of expression of virion protein) perform regulatory functions essential for virus replication. The accessory proteins nef (negative factor), vif (virion infectivity), vpr and vpu (viral proteins R and U) modulate virus replication and are essential for efficient virus reproduction in vivo and the resulting pathogenesis (reviewed in (Frankel and Young 1998; Freed 2001; Peterlin and Trono 2003)).

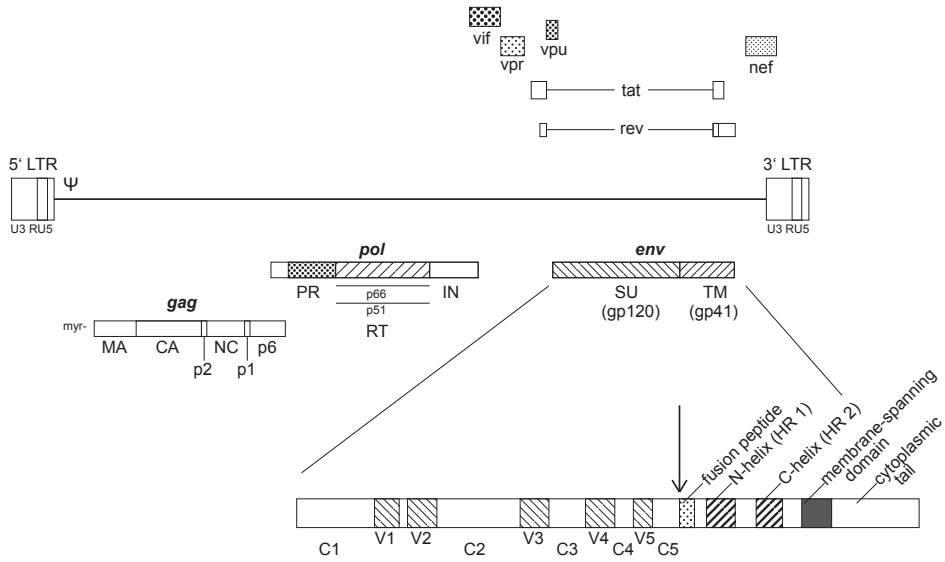

Figure 1 Organisation of the HIV genome. The relative location of the HIV-1 open reading frames gag, pol, env, vif, vpr, vpu, nef, tat and rev are indicated. The major gag domains (MA,CA,NC,p6) are shown under the gag gene. Under the pol gene the PR, RT and IN coding regions are indicated. The SU (gp120) and TM (gp41) env glycoproteins are enlarged to show the position of the different regions. Adapted from Freed 2001 with permissions from Springer's Copyright Clearance Center.

The outer viral envelope consists of a host-cell derived lipid bilayer containing various host membrane proteins (Ott 2008) and the viral envelope glycoproteins (Env) (Allan, Coligan et al. 1985) which are essential for attachment and entry (Chapter 3.3. HIV Attachment and Entry). The functional envelope spike of HIV consists of a trimer composed of hetero-dimers of the transmembrane protein gp41 non-covalently linked to the surface protein gp120 (Wyatt and Sodroski 1998).

Gp120 is a highly glycosylated protein (Poignard, Saphire et al. 2001) which contains a receptor site for the CD4 molecule (CD4-binding site; CD4bs) and a second site for binding to the co-receptors, usually CCR5 (Deng, Liu et al. 1996) or CXCR4 (Feng, Broder et al. 1996). Based on sequence analyses from different HIV isolates gp120 is divided into five conserved (C1-C5) and five variable (V1-V5) regions segments (Willey, Rutledge et al. 1986; Modrow, Hahn et al. 1987; Pantophlet and Burton 2006) (Figure 1 Organization of the HIV genome). Gp41 is composed of three major domains, namely the ectodomain, the transmembrane anchor sequence and the cytoplasmic tail (Chan, Fass et al. 1997; Weissenhorn, Dessen et al. 1997). It does not only anchor the gp120/gp41 complex in the membrane but also contains crucial domains for the membrane fusion process during entry (reviewed (Wilen, Tilton et al. 2012)). As the envelope spike is the only viral component in the outer viral envelope it represents a prime target for the humoral immune response of the host. However, HIV has ingeniously evolved several measures to effectively counteract the neutralizing antibody response, as will be shortly described in Chapter 3.4.1.

The inner layers of the mature virion- a highly organized macromolecular assembly- are formed within newly released virions upon proteolytic cleavage of the precursor p55 Gag polyprotein into the four gag splice proteins (MA, CA, NC and p6) (Figure 2 Virion Structure). This proteolytic processing generates the cleavage product MA (matrix, p17) that builds a matrix-layer and lines the inner viral membrane ensuring the integrity of the virion. CA (capsid, p24) assembles into the cone-shaped core, which encloses the viral RNA and virus encoded enzymes, which are needed for the generation and integration of the proviral DNA, namely reverse transcriptase (RT), integrase (IN) and protease (PR). NC (nucleocapsid, p7) tightly coats the viral RNA genome, two copies of positive single-stranded RNA, by forming the nucleocapsid-layer and stabilizes the RNA as ribonucleoprotein complex (reviewed in (Briggs and Kräusslich 2011).

The Vif protein is essential for viral replication in human CD4+ T cells (Simon, Gaddis et al. 1998) as it efficiently prevents the action of APOBEC3G, a dominant negative factor expressed in primary cells, by its ubiquitination (Mariani, Chen et al. 2003; Marin, Rose et al. 2003; Sheehy, Gaddis et al. 2003; Yu, Yu et al. 2003). In absence of Vif, APOBEC3G deaminates DNA:RNA hybrids, which ultimately leads to hypermutation of viral genes and genetic elements, resulting in their irreversible alteration and degradation (Sheehy, Gaddis et al. 2002; Mangeat, Turelli et al. 2003; Turelli and Trono 2005).

The Nef protein possesses most complex activities, which have been extensively documented (Foster and Garcia 2008). It is known to down-regulate CD4 (Garcia and Miller 1991; Aiken, Krause et al. 1996; Lundquist, Tobiume et al. 2002), as well as the MHC I and II molecules (Schwartz, Marechal et al. 1996; Wonderlich, Williams et al. 2008). Additionally it mediates cellular signaling and activation (Renkema, Manninen et al. 1999; Arora, Molina et al. 2000; Simmons, Aluvihare et al. 2001) and also enhances viral particle infectivity by CD4 independent mechanisms (reviewed in (Foster and Garcia 2008)).

The Vpu protein is involved in the release of new virions from infected cells (Strebel, Klimkait et al. 1988; Terwilliger, Cohen et al. 1989). It has been shown to counteract BST2 (also known as tetherin) which, in absence of Vpu, impedes viral particle release. Additionally, Vpu is known to act on newly synthesized CD4 molecules in the ER (Willey, Maldarelli et al. 1992) and subsequently to effect its catabolism (reviewed in (Dube, Bego et al. 2010)).

The Vpr protein is known to be crucial for efficient viral infection of CD4+ T cells and macrophages. This multifunctional accessory protein has numerous functions such as nuclear import of the HIV-1 preintegration complex, induction of the G_2 cell cycle arrest and modulation of T cell apoptosis (reviewed in (Le Rouzic and Benichou 2005; Kogan and Rappaport 2011)).

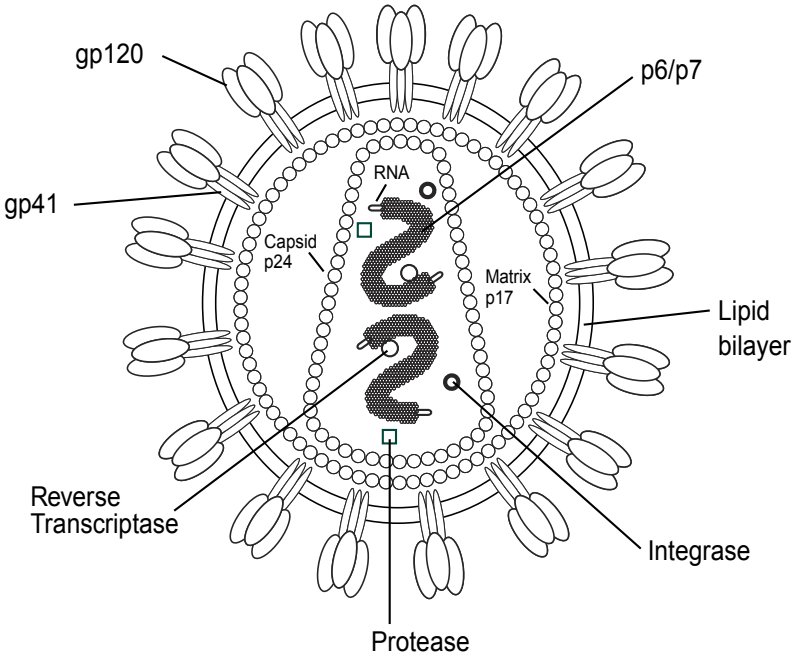

Figure 2 Virion structure. In HIV-1, trimeric gp120–gp41 complexes are embedded in the membrane. The transmembrane glycoprotein gp41 and the external envelope glycoprotein gp120 are depicted in non-covalent association. The capsid protein, p24, makes up the cone-shaped core, which contains two positive-strand RNA copies of the HIV-1 genome that are surrounded by the nucleocapsid protein (p7). Adapted from US National Institute of Health, Wikimedia commons.

3.3. HIV-1 Attachment and Entry

HIV-1 efficiently infects CD4+ target cells e.g. T-helper lymphocytes, macrophages, and dendritic cells. In order to infect and replicate inside its host, HIV follows an intricate and chronological sequence of events to enter these primary target cells. Virion binding to the cell surface is often facilitated by non-covalent interactions between Env and cell attachment factors such as heparin sulphate and mannose-binding lectins (e.g. DC-SIGN), which are not required for infection but improve virus-cell interactions (Mondor, Ugolini et al. 1998; Ugolini, Mondor et al. 1999; Baribaud, Pohlmann et al. 2001; Pohlmann, Baribaud et al. 2001) and increase trans-infection of T cells (Geijtenbeek, Kwon et al. 2000). Specific binding of the CD4 cell receptor to the highly conserved CD4-binding site of gp120 initiates an ordered multistep entry process (Dalgleish, Beverley et al. 1984; Wilen, Tilton et al. 2012) (Figure 3 HIV entry schematic). This interaction activates a conformational change in gp120 (Sattentau and Moore 1991; Kwong, Wyatt et al. 1998; Wyatt, Kwong et al. 1998) which subsequently induces exposure of the coreceptor binding site and enables binding of one of the co-receptors- predominantly the chemokine receptors CXCR4 (Feng, Broder et al. 1996) or CCR5 (Deng, Liu et al. 1996; Dragic, Litwin et al. 1996).

As HIV readily undergoes receptor activated conformational changes, the native envelope conformation has been suggested to be metastable and to transform upon receptor binding to an energetically more stable, fusion active conformation (Chan and Kim 1998). Coreceptor binding leads via a so-called “cast-and-fold” membrane fusion mechanism to a major conformational rearrangement in the gp41 trimer (Melikyan 2008). The N-terminal gp41 fusion peptide is exposed and projected toward the host membrane, followed by its insertion in the target cell membrane. This relatively stable conformational state, denominated “pre-hairpin intermediate”, exposes the two helical regions of the gp41 ectodomain, the N-terminal heptad repeat region (HR1) and C-terminal heptad repeat region (HR2) (Weissenhorn, Dessen et al. 1997; Chan and Kim 1998). From this pre-hairpin intermediate, the C-terminal HR2 fold back in an antiparallel fashion into the exterior grooves formed at the external interface of the three N-terminal HR1, resulting in a six-helix bundle formation (6HB) (Chan, Fass et al. 1997; Weissenhorn, Dessen et al. 1997). The formation of this highly stable 6HB stabilizes the nascent fusion pore and facilitates its expansion (Markosyan, Cohen et al. 2003). It has been predicted to be rate-limiting for fusion and to form in a stepwise process, releasing a fraction of the total free energy during each step to drive a series of energetically unfavorable lipid rearrangements that are necessary for membrane fusion (Melikyan, Markosyan et al. 2000; Markosyan, Leung et al. 2009) (reviewed in (Wilen, Tilton et al. 2012)).

Until recently HIV entry events were considered to occur at the cell surface supported by the observation that endocytic internalization and endosomal acidification appear not to be required for HIV entry into the cytoplasm (Stein, Gowda et al. 1987; Kielian and Jungerwirth 1990; Skehel and Wiley 2000). However, different cell types (eg macrophages, epithelial cells, lymphoid cells)

have been observed to internalize and harbor HIV particles in vesicular structures (Fackler and Peterlin 2000; Marechal, Prevost et al. 2001; Blanco, Bosch et al. 2004; Schaeffer, Soros et al. 2004). A seminal study by Miyauchi et al. revealed that HIV-1 infects cells via an envelope glycoprotein- and dynamin-dependent fusion with intracellular compartments (Miyauchi, Kim et al. 2009). As new imaging technologies and approaches to block different pathways of HIV entry are constantly providing new insights into the potential role of endocytic entry in HIV replication, the original paradigm that HIV-1 fuses predominantly at the cell surface must be questioned.

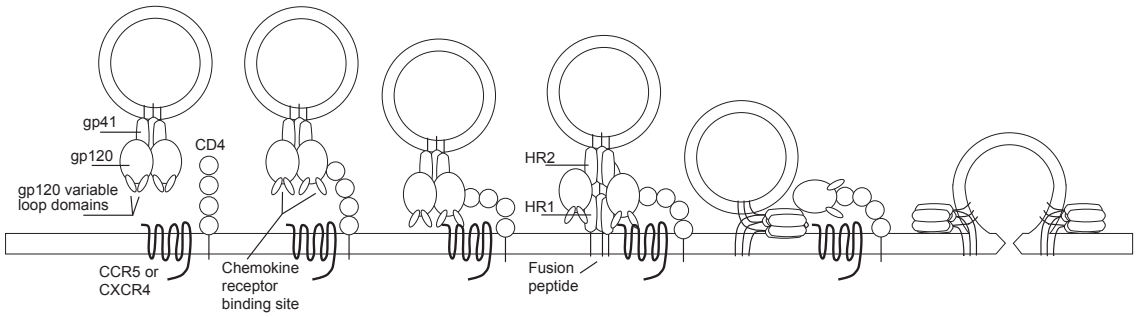

Figure 3 HIV entry schematic. The well defined HIV fusion process leading to delivery of the HIV viral core into the cytoplasm begins with the binding of the viral envelope glycoprotein gp120 with the cellular receptor CD4. After gp120-CD4 engagement, conformational changes in gp120 trigger its interaction with the viral chemokine receptor CXCR4 or CCR5. Thereafter, gp41 is exposed into a fusogenic conformational stat that triggers the insertion of the fusion peptide into the target cell. Finally the six helix bundle formation stabilizes and enlarges the nascent fusion pore. Modified from (Moore and Doms 2003; Tomkowicz and Collman 2004).

3.4. HIV-1 Entry Inhibition

3.4.1. Humoral immune response against HIV-1

The antibody response to HIV can be detected within few weeks (Moore, Cao et al. 1994; Mascola and Montefiori 2010) in plasma of HIV infected individuals and is predominantly directed against the structural proteins such as the envelope proteins gp120, gp41 as well as the core capsid (p24) and matrix (p17) (Belec, Dupre et al. 1995; Binley, Klasse et al. 1997; Richman, Wrin et al. 2003; Aasa-Chapman, Hayman et al. 2004).

Neutralizing activity is attributed to antibodies directed against specific epitopes on the envelope glycoproteins gp120 and gp41 that are exposed in the oligomeric form of the HIV envelope protein (Parren, Moore et al. 1999; Burton, Stanfield et al. 2005; Huber and Trkola 2007). However, the majority of antibodies directed against the viral envelopes recognizes non-neutralizing epitopes of glycoprotein monomers and are therefore ineffective (Burton 1997; Wyatt, Kwong et al. 1998; Parren, Moore et al. 1999). This can be explained with the elaborate mechanisms HIV has evolved to avoid recognition by the humoral immune system. Gp120 is heavily glycosylated by the host cell glycosylation machinery, which provides a so-called glycan shield for the envelope and avoids immune system recognition (Leonard, Spellman et al. 1990; Wei, Decker et al. 2003; Pantophlet and Burton 2006). Additionally, the conserved functional domains of the envelopes are masked by variable loops and only transiently exposed during viral entry (Johnson and Desrosiers 2002; Kwong, Doyle et al. 2002). Also, HIV's enormous sequence variability due to the high mutation rate of RT transcription (Drake 1993) is an effective mechanism to evade humoral immune response pressure. Sequence variability is concentrated mainly in the variable, flexible loop structure of gp120 (Wyatt, Kwong et al. 1998; Pinter 2007).

The small fraction of HIV neutralizing antibodies (NAbs) isolated so far have been shown to interfere with virion attachment to CD4 or to inhibit post-attachment entry steps (Trkola, Dragic et al. 1996; Wyatt and Sodroski 1998; Parren and Burton 2001; Pantophlet and Burton 2006; Huber and Trkola 2007; Alam, Morelli et al. 2009). Most of the NAbs are specific to the autologous virus and will not neutralize viral isolates from other patients (Wrin, Crawford et al. 1994; Richman, Wrin et al. 2003). Nevertheless, at later stages of chronic infection a small fraction (10%–30%) of patients will develop antibodies which successfully inhibit a variety of virus isolates, so-called broadly neutralizing antibodies (bNAbs) (Doria-Rose, Klein et al. 2009; Sather, Armann et al. 2009; Simek, Rida et al. 2009; Mikell, Sather et al. 2011). These bNAbs target epitopes on HIV env that have a crucial role in viral attachment and entry and are thus conserved across such a broad range of isolates (Deeks, Schweighardt et al. 2006; Pantophlet and Burton 2006; Mikell,

Sather et al. 2011). So far, only very few bNAbs have been isolated from patients (see “Figure 4 Broadly neutralizing antibodies” for an overview), but recent advances in antibody isolation technology have accelerated the identification of new bNAbs (Kwong, Mascola et al. 2009; Wu, Yang et al. 2010; Scheid, Mouquet et al. 2011).

CD4bs directed antibodies: b12 and VRC01

The binding site for CD4 on gp120 is a highly conserved region crucial for virus attachment (Kwong, Wyatt et al. 1998; Wyatt, Kwong et al. 1998). A prime target for NAbs due to its conservation and requirement for accessibility, the CD4-binding site is protected from the humoral response through glycan shield and conformational masking (Chen, Do Kwon et al. 2009). Until recently, the bNAb b12 was the broadest and most potent CD4bs-directed antibody described (Barbas, Bjorling et al. 1992; Burton, Pyati et al. 1994; Binley, Wrin et al. 2004; Stamatatos, Morris et al. 2009). Its most prominent feature is the complementarity determining region 3 (CDR H3), which is relatively long compared with the average human antibodies (Saphire, Parren et al. 2001; Burton, Stanfield et al. 2005). A new CD4bs-directed antibody, VRC01, displaying even a greater breadth and potency compared to b12 has been isolated lately (Wu, Yang et al. 2010; Zhou, Georgiev et al. 2010). It has an unremarkable CDR H3 but with extensive somatic mutations from affinity maturation (Wu, Yang et al. 2010; Verkoczy, Kelsoe et al. 2011).

V3 loop directed antibody: 447-52D

Despite it variation in sequence between different virus isolates, the V3 loop of gp120 contains a relatively conserved sequence motif GFPR or GPGQ that may be important for binding to the co-receptor (Hartley, Klasse et al. 2005). The V3 loop specific antibody 447-52D neutralizes a variety of isolates that bear the GPGR motif and retains within the subtype B a relatively broad reactivity (Binley, Wrin et al. 2004; Zolla-Pazner, Zhong et al. 2004).

Glycan specific antibody: 2G12

The bNAb 2G12 binds a cluster of high mannose carbohydrates of N-linked glycosylated amino acid residues of gp120 (Trkola, Purtscher et al. 1996; Sanders, Venturi et al. 2002; Scanlan, Pantophlet et al. 2002). As glycans are attached by the host glycosylation machinery, they usually are non-immunogenic. The explanation how 2G12 recognizes glycans exclusively has been found

in its unique structure in which the two Fabs of the IgG assemble into interlocked VH domains-swapped dimer (Calarese, Scanlan et al. 2003; Burton, Stanfield et al. 2005). This extraordinary structure provides an extended interaction with a conserved cluster of oligomannoses on gp120. 2G12 has been shown to have broad neutralizing activity against isolates of subtype B and minor activity against other subtypes (Burton, Pyati et al. 1994; Trkola, Pomales et al. 1995; Binley, Wrin et al. 2004; Trkola, Kuster et al. 2005).

MPER specific antibodies: 2F5 and 4E10

The antibodies 2F5 (Muster, Steindl et al. 1993; Purtscher, Trkola et al. 1994; Trkola, Pomales et al. 1995) and 4E10 (Stiegler, Kunert et al. 2001; Zwick, Labrijn et al. 2001) bind to the highly conserved membrane proximal external region (MPER),a tryptophan-rich region immediately adjacent to the membrane-spanning domain, of gp41(Salzwedel, West et al. 1999). It has been shown, that the MPER domain becomes more accessible, once gp120 has bound cellular receptors and envelope rearrangements proceed, allowing the antibodies to rapidly bind and neutralize the virus (Binley, Cayanan et al. 2003; Cardoso, Brunel et al. 2007; Frey, Peng et al. 2008; Alam, Morelli et al. 2009; Buzon, Natrajan et al. 2010; Frey, Chen et al. 2010). Cross-reactivity of the CDR H3 loop of 2F5 with membrane lipids has proven important for the potent neutralization activity of 2F5 (Haynes, Fleming et al. 2005; Sanchez-Martinez, Lorizate et al. 2006; Ofek, McKee et al. 2010). A recent study has demonstrated the capacity of MPER antibodies to irreversibly sterilize HIV virions by induction of gp120 shedding, which is closely associated with MPER antibody inhibition (Ruprecht, Krarup et al. 2011).
In a clinical study, combination of 2G12, 2F5 and 4E10 was able to delay viral rebound in several HIV-1-infected individuals undergoing interruption of antiretroviral treatment (ART). (Trkola, Kuster et al. 2005). Notably, escape mutant analysis revealed that the activity of 2G12 was crucial for the in vivo effect of the neutralizing antibody cocktail (Trkola, Kuster et al. 2005).

Recent mAb discoveries: PG9, PG16 and PGT mAb

Using a high-throughput functional screening approach, two new, somatically related bNAbs, PG9 and PG16, were isolated from a clade A infected donor (Kwong, Mascola et al. 2009; Walker, Phogat et al. 2009). They recognize a quaternary site on gp120 composed of elements in the V1/V2 of gp120 that is present on the trimeric HIV-1 Env. A very recent study has identified a new group of bNAbs, named PGT, that exhibited cross-clade neutralizing activity and showed extraordinary potency (Walker, Huber et al. 2011). The PGT monoclonal antibodies target two

conserved glycans as well as a short β-strand segment of the gp120 V3 loop, which is assumed to be responsible for their high binding affinity and broad specificity (Pejchal, Doores et al. 2011).

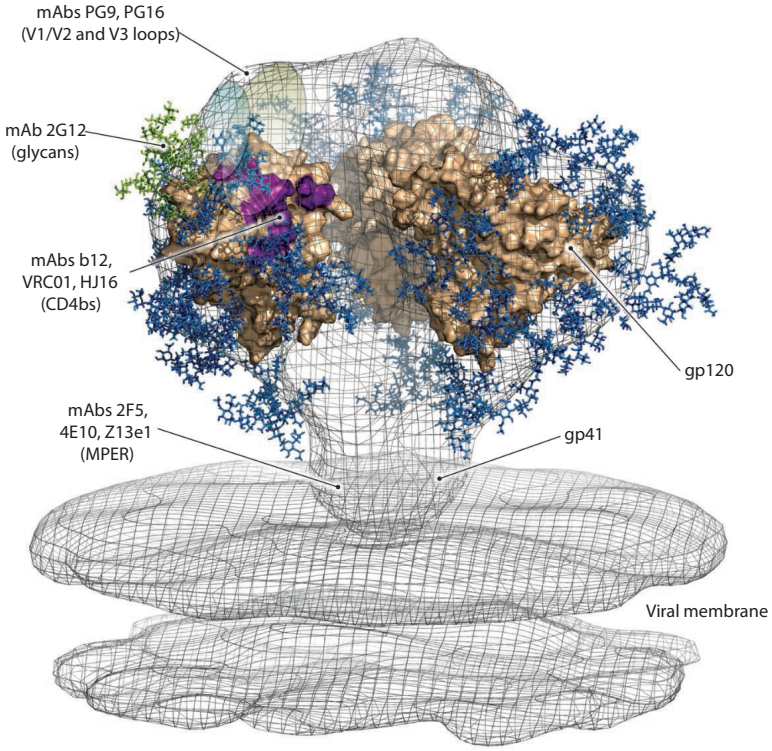

Figure 4 Broadly neutralizing antibodies. Broadly neutralizing monoclonal antibodies (mAbs) target epitopes on the viral envelope spike at the surface of HIV-1. The spike is a heterotrimer containing the viral glycoproteins $(gp120)_3$ $(gp41)_3$. This model was generated by Burton et al. by combining cryoelectron tomographic, crystallographic, and computational analyses. The gp120 core structure (tan), fitted in the electron density map (gray) of the spike, the membrane proximal external region (MPER) and viral membrane are represented. The V1/ V2 and V3 protein loops are shown as ovals (light green and light blue) at the top of the spike. Glycans (green and blue) are indicated. From (Burton and Weiss 2010). Reprinted with permission from AAAS.

3.4.2. Entry inhibitors

Entry of HIV-1 into host cells presents the first target of the HIV-1 life cycle for therapeutic intervention. Therefore several attempts have been made to find an effective inhibitor for this crucial step of viral spread. Drugs that block HIV-1 entry are collectively defined as entry inhibitors, but compose a multifaceted group of drugs with different mechanisms of action. This variability reflects the multi-step process of HIV-1 entry comprising from attachment to the host cell to membrane fusion. Generally, the group can be subdivided into three classes of agents that inhibit different steps of entry: first attachment and CD4 binding, then coreceptor engagement and last membrane fusion. So far only two entry inhibitors have been approved by the food and drug administration FDA for HIV treatment: the CCR5 inhibitor Maraviroc (Celsentri®) (Dorr, Westby et al. 2005) and the fusion inhibitor T-20 (Fuzeon®) (Wild, Greenwell et al. 1993).

3.4.2.1. Inhibitors of the gp120-CD4 interaction

Early attempts to develop specific inhibitors of HIV-1 entry focused on the design of recombinant soluble CD4 molecules, which lack the transmembrane and cytoplasmic domains of CD4, but efficiently bind gp120. Soluble CD4 (sCD4) was found to inhibit T- cell laboratory adapted strains potently in vitro (Moore 1990), but did not reduce viral loads in HIV infected patients, which was later found to be due to a dramatically reduced activity against primary HIV isolates (Daar, Li et al. 1990). More promising data results were achieved with CD4-IgG2 (PRO 542) (Allaway, Davis-Bruno et al. 1995), which is a tetravalent fusion protein that consists of the domains D1 and D2 of human CD4 fused to the Fc portion of human IgG2. It has been found to induce a short lived active state of gp120 that converts into a non-functional conformation and thereby blocks viral entry. PRO 542 was found to broadly and potently neutralize primary HIV-1 isolates in vitro (Trkola, Pomales et al. 1995; Trkola, Ketas et al. 1998). However, only modest reduction in a phase II trial in plasma HIV-1 RNA levels were reached in patients with advanced HIV disease (Jacobson, Lowy et al. 2000; Jacobson, Israel et al. 2004).

The small molecule inhibitor BMS-378806, which binds the conserved CD4-binding site of gp120, (Wang, Zhang et al. 2003) seemed to be most promising as it showed some favourable pharmacokinetic traits in animal models and a good safety profile in preclinical testing (Lin, Blair et al. 2003). Unfortunately, it failed to achieve targeted plasma concentration (Xue, Yan et al. 2007) and showed limited clinical utility due to low genetic barrier to resistance (Lin, Blair et al. 2003), whereupon the development was halted (Hanna, Lalezari et al. 2011). However, the related inhibitor BMS-488043 (Yang, Zadjura et al. 2010) has improved in vivo performance reducing plasma HIV-1 RNA by greater than 0.7 $\log_{10}$ copies/ml within 8 days, suggesting that further development of this novel class of oral HIV-1 attachment inhibitors is still warranted (Hanna, Lalezari et al. 2011).

The CD4 miniprotein mimetic CD4M47 has been developed by transplanting the gp120-binding surface of CD4 into a toxin scaffold (Drakopoulou, Vizzavona et al. 1998; Vita, Drakopoulou et al. 1999). CD4M47 displays a nanomolar affinity for gp120 and crystallographic analysis revealed extensive mimicry between the binding surfaces on the CD4M47 and CD4 for gp120, as well as between the CD4M47- and CD4-induced conformations of gp120 (Stricher, Huang et al. 2008). CD4M47 potently inhibits HIV entry in vitro but whether this type of inhibitor possesses activity in vivo still needs to be investigated (Martin, Stricher et al. 2003; Stricher, Huang et al. 2008).

An additional strategy for blocking the interaction between CD4 and gp120 is to directly target the CD4 cell receptor. The attempt to find an inhibitor against the host receptor CD4 is an alternative strategy as it targets broadly divergent strains and is less affected by the high genetic variability of HIV. By employing the Designed Ankyrin Repeat Protein (DARPin) technology CD4-specific DARPins were selected which potently block HIV entry (Schweizer, Rusert et al. 2008). DARPins are an effective alternative to antibodies as they bind any target with high affinity and specificity

but are clearly superior in terms of physical stability and production costs (Binz, Amstutz et al. 2005). The CD4-specific DARPins have been shown to be broadly active inhibitors of HIV entry in vitro and may represent a novel type of inhibitor molecules in HIV infection (Schweizer, Rusert et al. 2008). Most monoclonal antibodies targeting CD4 were found to be immunosuppressive as they blocked the CD4-MHC II interaction (Delmonico and Cosimi 1996) and hence development had to be halted. However, ibalizumab (formerly known as TNX-355), a humanized IgG4 mAb, targeting domain 2 of human CD4 does not prevent attachment to MHC II or gp120. Yet, it successfully inhibits HIV-1 entry by blocking CD4 induced conformational changes in gp120 (Kuritzkes, Jacobson et al. 2004). In HIV patients, single as well as multiple doses of ibalizumab caused substantial reduction (~2$\log_{10}$) in viral loads and increase in CD4 count without any indication to serious adverse effects or immunologic impairments (Kuritzkes, Jacobson et al. 2004; Jacobson, Kuritzkes et al. 2009).

3.4.2.2. Inhibitors of the gp120- coreceptor interaction

Despite the rapid identification of CD4 cell as the “primary receptor” for HIV (Dalgleish, Beverley et al. 1984), it soon became clear that additional molecules might be involved. CXCR4 was identified as the coreceptor for X4 HIV-1 isolates (Feng, Broder et al. 1996) followed by the discovery of the coreceptor CCR5 for R5 HIV-1 isolates (Alkhatib, Combadiere et al. 1996; Choe, Farzan et al. 1996; Deng, Liu et al. 1996; Dragic, Litwin et al. 1996). The identification of a subset of individuals highly resistant to HIV-1, which were found to be homozygous for an inactivating deletion for CCR5, Δ32-CCR5 (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996), led to the conclusion that an inhibition of the gp120-CCR5 interaction may be an effective strategy for HIV-1 therapy.

A number of different inhibitors targeting the gp120-coreceptor interaction have been developed, such as derivatives from the naturally occurring chemokine ligands which competitively antagonize CCR5 binding, small molecule inhibitors which induce conformational alterations in CCR5 therefore act as allosteric inhibitors and antibodies that block the CCR5 receptor without interfering with ligand binding.

The naturally occurring CCR5 ligands CCL3 (MIP1α), CCL4 (MIP-1β) and CCL5 (RANTES) (Cocchi, DeVico et al. 1995) inhibit HIV-1 infection by binding to the receptor, which leads to the occlusion of gp120 binding site, as well as by inducing its internalization (Alkhatib, Locati et al. 1997; Trkola, Paxton et al. 1998). One of the several RANTES derivatives, PSC-RANTES (Lederman, Veazey et al. 2004), is currently in development as a potential microbicide (Mintakafoundation 2011). Even though PSC-RANTES has been proven to be fully protective when applied topically in a macaque model of vaginal HIV transmission (Lederman, Veazey et al. 2004), its high produc-

tion costs and strong CCR5 agonist activity might induce local inflammation and hinder the future clinical application.

Many small molecules antagonists have demonstrated efficacy against HIV replication in vitro. Maraviroc (UK-427857), a small-molecule antagonist binding to the hydrophobic pocket in the transmembrane helices of CCR5 (Dorr, Westby et al. 2005), was approved in 2007 by the FDA for combination antiretroviral treatment of adults infected with only CCR5-tropic HIV and with CCR5 using HIV strains resistant to multiple antiretroviral agents (FDA 2011). SCH-350581 (AD101) (Tsamis, Gavrilov et al. 2003) and SCH-C (Tsamis, Gavrilov et al. 2003), two structurally related compounds, inhibit CCR5-mediated viral infection in a nanomolar range. AD101 and SCH-C bind to the TM domain of CCR5 and disrupt the conformation of the extracellular domain, thus inhibiting the binding of chemokines, MAbs, and gp120 to CCR5 (Strizki, Xu et al. 2001; Seibert, Ying et al. 2006). SCH-C had to be discontinued due to adverse effects, but Vicriviroc (SCH-D, SCH-417690) (Strizki, Tremblay et al. 2005), a second generation compound based on SCH-C, has just completed phase II trials (ClinicalTrials.gov 2011). PRO-140, a humanized CCR5- directed antibody has proven to inhibit HIV potently both in vitro and in HIV patients (Trkola, Ketas et al. 2001; Jacobson, Saag et al. 2008) and is currently in a phase II b trial (ClinicalTrials.gov 2011). It binds to an extracellular epitope on CCR5 acting as a competitive inhibitor of virus binding and therefore, unlike the available small molecule CCR5 inhibitors, antiviral concentration of PRO140 do not block natural function of CCR5 in vitro (Olson, Rabut et al. 1999).

In contrast to CCR5, CXCR4 is essential for multiple physiological processes (Tachibana, Hirota et al. 1998; Ma, Jones et al. 1999) and its deletion resulted in abnormal cerebral development and was embryonically lethal in a mice knockout study (Tachibana, Hirota et al. 1998; Zou, Kottmann et al. 1998). The different ligand derivatives and small-molecule antagonists targeting CXCR4 have either failed to show any significant viral load reduction or their development has been halted due to adverse effects (reviewed in (Tilton and Doms 2010)).

3.4.2.3. Inhibitors of membrane fusion

Fusion inhibitors were the first entry inhibitors to be approved for HIV-1 infection and have shown sustained effectiveness. Synthetic peptides corresponding to the HR1 and HR2 domains of gp41 (Wild, Oas et al. 1992; Jiang, Lin et al. 1993) - unintentionally discovered during a screening for vaccine targets - were found to have potent antiviral effects by inhibiting the formation of the six-helix bundle (Chan, Fass et al. 1997; Weissenhorn, Dessen et al. 1997). The fusion inhibitor enfurtivide (T-20) - a linear, 36 amino acid long peptide with a sequence identical to part of the HR2 region of gp41 (Wild, Greenwell et al. 1993)- showed high potency in clinical trials (Kilby, Lalezari et al. 2002; Lalezari, Eron et al. 2003). A decade after its discovery, T-20 was approved in 2003 for clinical application by the FDA for treatment experienced HIV positive patients (FDA 2011).

3.5. HIV-1 cell-cell transmission

The viral spread by direct movement of viruses between contacting cells has been shown to be important for several mammalian genera of virus, eg Flaviviruses (HCV (Timpe, Stamataki et al. 2008)), Herpesviruses (eg VZV (Reske, Pollara et al. 2007) and Paramyxoviruses (eg RV (Rima and Duprex 2006). The increasing knowledge on the mechanism of viral dissemination has revealed that these families have adapted cell-cell spread advantageously in a number of specific ways (reviewed in (Johnson and Huber 2002; Sattentau 2008). The transmission without use of long-distance fluid phase diffusion has also been proven to be very efficient for Retroviruses. One of the first studies addressing this question showed that HIV-1 is able to efficiently spread from one cell to an adjoining uninfected cell within minutes (Sato, Orenstein et al. 1992). These findings were supported by microscopic analysis revealing virus particles in points of cell contiguity between Macrophages and T cells (Carr, Hocking et al. 1999). Even though early publications described CD4 and adhesion molecule (such as ICAM-1; Intercellular Adhesion Molecule 1) polarization upon gp120 binding (Fais, Capobianchi et al. 1995; Iyengar, Hildreth et al. 1998), it was first shown for HTLV-1 by Igakura et al. that the junction formed between infected and uninfected cells had a highly ordered architecture containing talin domains and a polarized microtubule organizing center (MTOC) (Igakura, Stinchcombe et al. 2003) (Figure 4 Scheme of virological synapse). Seminal papers showed subsequently that Dendritic Cell-T-cell contacts facilitate HIV-1 transfer by locally concentrating virus, receptor and coreceptors during the formation of points of contiguity (McDonald, Wu et al. 2003) and revealed a gp120 dependent recruitment of CD4, CXCR4 and LFA-1 (Lymphocyte Function-associated Antigen 1) to the interface in an actin-dependent mechanism (Jolly, Kashefi et al. 2004). Intriguingly, despite the fact that the lifespan of infected donor- uninfected target cell conjugates had been shown to last several hours, only limited cell-cell fusion was observed (Jolly, Kashefi et al. 2004; Chen, Hubner et al. 2007; Martin, Welsch et al. 2010). In line with these observations, the interaction between an infected donor and uninfected target cell was termed ”Virological Synapse” ((Jolly and Sattentau 2004) reviewed in (Sattentau 2010)).

The resemblance of the virological and the immunological synapse -a receptor containing adhesive junctions important for the T cell -T cell communication- was underlined by latest studies showing that viral protein nef actively hijacks the immunological antigen presenting system to efficiently propagate HIV spread between cells (Nobile, Rudnicka et al.) by triggering existing pathways involved in antigen presentation and T cell-T cell communication (McMichael, Borrow et al. 2010). Moreover, HIV-1 is able to promote cell-cell spread by also modulating innate immunity (reviewed in (Jolly 2011)). HIV-1 annuls the first line of the cellular innate response by targeting mature myeloid dendritic cells and promoting trans-infection of CD4+ T cells (Geijtenbeek, Kwon et al. 2000; McDonald, Wu et al. 2003; McDonald 2010). It has also been shown that T-cell-T-cell

transmission is less sensitive to interferon-mediated inhibition than cell-free infection (Vendrame, Sourisseau et al. 2009). Additionally, HIV cell-cell transmission overcomes efficiently entry restriction by rhesusTRIM5α (Richardson, Carroll et al. 2008) and cannot be inhibited by endogenous tetherin (Jolly, Booth et al. 2010), showing that this efficient mode of transmission abrogates the function of different host restriction factors.

Recent publications have shown that transmission occurs not only through close contacts but also via long intercellular tubular structures termed “membrane nanotubes”, which emanate from infected and uninfected T cells and join in a “micro-synapse” (Sowinski, Jolly et al. 2008; Rudnicka, Feldmann et al. 2009), and filopodia (Sherer, Lehmann et al. 2007). This was underlined by the studies revealing that HIV-1 could not only congregate single interacting cell conjugates, but also “polysynapses” increasing the transmission efficiency (Hubner, McNerney et al. 2009; Rudnicka, Feldmann et al. 2009).

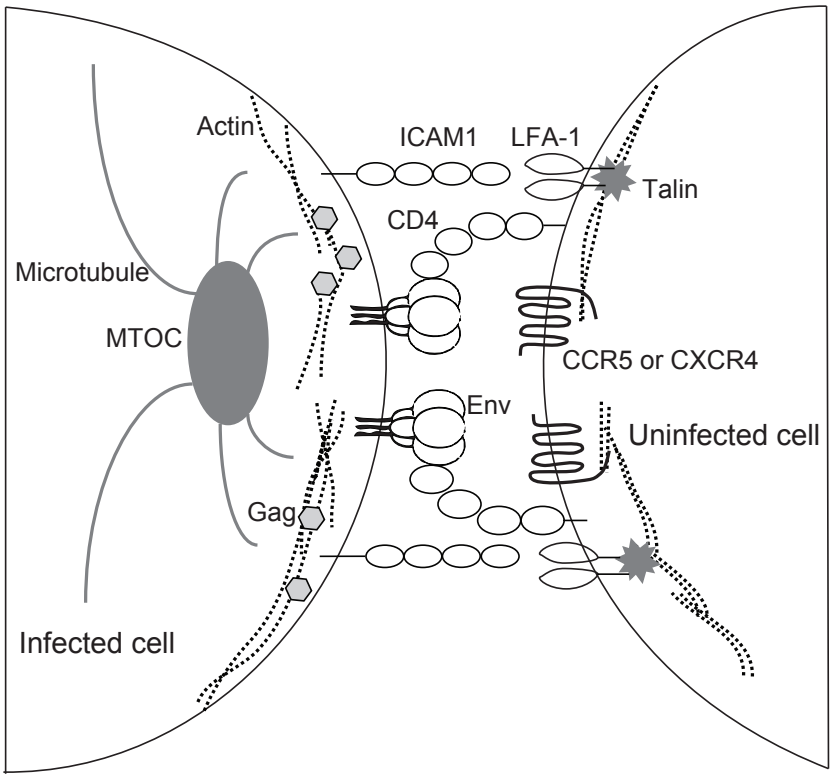

Figure 5 Schematic diagram of the virological synapse. The cell–cell contact zone contains tight junctions and a synaptic cleft into which virions are released from the infected cells. HIV-1 envelope glycoproteins (Env) are expressed on the infected cell plasma membrane and interact with the receptors CD4 and CCR5 or CXCR4 on the target cell. The adhesion molecules intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1) engage to stabilize the cellular conjugate (Jolly, Mitar et al. 2007). Figure based on results presented in (Igakura, Stinchcombe et al. 2003; McDonald, Wu et al. 2003; Jolly, Kashefi et al. 2004). Adapted from (Sattentau 2008) with permissions from Springer’s Copyright Clearance Center.

3.5.1. Neutralization of HIV-1 cell-cell transmission

The principal capacity of NAbs to block HIV spread has been studied extensively over the past two decades and was ascertained in vitro and in vivo, in animals models and in human passive immune transfer studies (Trkola, Kuster et al. 2005; Hessell, Poignard et al. 2009; Mascola and Montefiori 2010). However, to date it still remains unclear to what extent the relatively enclosed environment of the viral synapse is able to protect the virus from humoral immunity. A number of studies have shed light on the complexity of HIV transmission modes and revealed incongruities amongst the findings of cell-cell transmission neutralization.

An early study investigating the efficacy of neutralizing antibodies in inhibiting cell-cell transmission, suggested that HIV-1 T cell-T cell spread is rather resistant to general neutralizing monoclonal antibodies (Keele, Van Heuverswyn et al. 2006). In this line, the study by Ganesh et al. found a remarkable loss of inhibition by b12 during coculture of mature dendritic cells (mDC) infected with replication competent virus and T cells (Ganesh, Leung et al. 2004). In contrast, the investigation of Massanella et al. focusing on the antigen transfer between 293-T and CD4 T cells, described that early events of synapse formation were resistant to neutralizing antibodies, but detected efficient neutralization of later steps of virus transfer by the MPER antibodies 2F5 and 4E10 (Massanella, Puigdomenech et al. 2009). In line with these findings Monfort et al. reported that anti-gp41 specific NAb 2F5 was able to interfere with HIV-1 spread between T cells (van Montfort, Nabatov et al. 2007). A most recent study showed efficient neutralization by Nabs b12, 2G12 and 2F5 and did not detect any difference in susceptibility between cell-cell and cell-free transmission (Martin, Welsch et al. 2010). Considering receptor and coreceptor variability, it is not surprising that monitoring cell-cell transmission, precise quantification of the events, and assessment of inhibitor efficacy has remained most complex. In part conflicting results obtained on neutralizing antibody efficacy in blocking HIV cell-cell transmission may be a consequence of the variable types of cell-cell interactions engaged in contacts between cells of different origin as well as differential assay systems and readouts. A major drawback of the cell-cell transmission assays developed to date is the lack of dissection between cell-cell and cell-free transmission (Table 1“Comparison published cell-cell transmission assays”)

Cell-Cell transmission assays

	cell type				Detection of transmission events			Neutralization
Publication	donor cell (HIV+)	target cells (HIV-)	Virus	readout	cf only	cc only	mix of cc and cf	nAbs tested
Gupta et al. 1989, JVI	PBMC	PBMC	rc		+	-	+	-
Ganesh et al. 2004, JVI	mature DC	T-cell line (A3R5) or primary CD4+ T-cells	mDC infected with rc or transduced pp	i.c.p24 after 30min of coculture	+	indinavir present during coculture	+	+
				i.c.p24 after 48h of coculture				-
Jolly et al. 2004, JEM	Jurkat T cell line chronically inf. with LAI	purified primary CD4+ T cells from PBMC	rc	Microscopy	not shown	-	+	
Sourriseau et al. 2007, JVI	Jurkat Tcell line primary CD4 T cells	Jurkat Tcell line CFSE+ primary CD4 T cells	rc	FACS	+	-	+	
Hubner et al. 2009, Science	HIV Gag-iGFP-expressing Jurkat cells	purified primary CD4+ T cells from PBMC	rc	FACS and Microscopy	not shown	-	+	+
Van Montfort et al. 2009, AIDS	mature DC	Raji DcSIGN T-cells	rc	FACS	not shown	-	+	+
Massanella et al. 2009, AIDS	MOLT/CCR5 cells (chronically inf.)	CD4 T-cells	rc	FACS (cytoplasmic dye transfer) 2h post coculture		-		-
	MOLT/CCR5 cells (chronically inf.)	CD4 T-cells	rc	i.c. p24 FACS 24h post coculture	not shown	-	+	
	MOLT/CCR5 cells (chronically inf.)	CD4 T-cells	rc	real-time PCR after 24h; productive infection of target cell	not shown	-	+	+
Martin et al. 2010, JVI	Jkt BAL	A3.01	rc	measurement of de novo synthesized viral DNA by qPCR	+	early readout of cc; no cf infection	+	+

Table 1 Comparison published cell-cell transmission assays. This table gives a summary if the cell-cell transmission assays published so far. Abbreviations: PBMC, Peripheral blood mononuclear cells; rc, replication competent; cc, cell-cell transmission; cf, cell-free infection; NAbs, neutralizing Antibodies; i.c., intracellular stainng; FACS, Flow Cytometry

3.6. Therapeutic antibodies in HIV Treatment-Classical approaches to novel advances

Review published in Current Pharmaceutical Design

Therapeutic Antibodies in HIV Treatment - Classical Approaches to Novel Advances

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Abstract: Therapeutic antibodies have evolved into an important drug class and have achieved considerable success in combating cancers and autoimmune diseases. Although their potential in the treatment of viral infections has not yet been fully explored, recently established approaches have the potential to aid the development of HIV specific antibody therapies.

Antibody engineering has led to improvements in antibody isolation and increases in antibody efficacy and potency. Strategies have been developed to tailor Fc recruitment of effector functions, and conjugation of monoclonals to toxins endows them with the ability to mediate destruction of specific target cells.

These technical advances introduce the possibility of designing a therapy to target and clear cells infected with a broad range of HIV strains and recommend some hypothetical clinical settings in which advanced antibody therapeutics could be employed in prophylaxis or therapy for HIV infection.

Keywords: HIV, AIDS, therapeutic antibodies, passive immunization, infectious diseases.

INTRODUCTION

Throughout HIV infection considerable levels of virus specific antibodies are elicited; however, most are non-neutralizing and once neutralizing antibodies (nAbs) are elicited the virus rapidly evades this immune pressure (reviewed in [1]). A few broadly neutralizing antibodies have been identified [2-12] and proven to successfully protect against HIV infection upon passive immunization in small scale human and animal trials [13-24]. These antibodies have been models for the design of novel therapeutic interventions and vaccines. However, so far vaccine development has failed to induce sufficient antibody responses to mediate sterilizing immunity [25,26]. Despite efficiently suppressing viremia, current anti-HIV drug therapies do not eradicate the virus, and thus alternative strategies will be required to clear the infection [27,28]. As a consequence an unprecedented effort to define new viral and cellular targets for prevention of replication and transmission, and to eliminate the infected cell reservoirs, is ongoing.

Since lifelong administration of mAbs would probably be necessary to control disease progression the high cost of their production, and the restricted potential for self-administration, have limited motivation to develop antibody based treatments for HIV. However, recent advances in antibody engineering may overcome some of these hurdles and make monoclonal antibodies a more attractive therapeutic option.

Passive administration of mAbs alone will not be sufficient to clear a chronic infection like HIV, nor will it be appropriate as a prophylactic. In the case of continuous administration of a single nAb viral escape variants are likely to emerge sooner or later [29,30]. Equally polyclonal treatments would need to be regularly tailored to the patient's evolving viral population, rendering continuous nAb treatment unfeasible. Nevertheless, due to their high specificity, long half-life and capacity to elicit immune effector functions or deliver toxins mAbs may —in combination with current antiretroviral therapy— prove effective in some specific clinical settings. Abs could provide a valuable component in treatment strategies aiming to reactivate latent viral reservoirs and mediate specific destruction of HIV infected cells [31].

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In this review we briefly summarize established approaches to develop antibody therapeutics in diverse disease settings, and highlight recent findings that have the potential to facilitate the development of HIV specific antibody therapies. A particular emphasis is put upon the advances in antibody engineering and antibody isolation efficiency, increases of antibody efficacy and potency, mediation of effector functions, and antibody delivery. We also suggest hypothetical clinical settings in which advanced HIV antibody therapeutics could potentially be of use.

MECHANISMS OF ANTIBODY ACTIVITY IN VIRAL INFECTION

Significant vaccine-induced reductions of infectious diseases - particularly viral diseases (polio, measles, mumps, rubella, hepatitis B, and influenza virus) - have been achieved since the first successful immunizations against smallpox in the 18th century [32]. Since it has been recognized that intrinsic neutralizing antibody responses provide protection in a number of viral diseases the primary goal of vaccine development has been to build an immunological barrier by producing neutralizing antibodies. At best these will prevent infection or, if not, at least reduce the severity of symptoms [32]. Notably, antibody based vaccines have proven most effective against viral pathogens that establish transient, acute infections and are commonly cleared. In comparison vaccines against persistent infections have achieved limited success. Consequently it is generally predicted that protection against HIV will require both elicitation of humoral and cellular immunity (reviewed in [33]).

Passive immunization was one of the first therapies against bacterial toxins in the early 1890's and - due to its high tolerability and efficacy in treating non-persisting infections - has been widely used against a variety of microbial infections (reviewed in [34]). Despite tremendous efforts in drug development, adoptive transfer of monoclonal or polyclonal immunoglobulin has remained a standard of care for the prophylaxis and treatment of a variety of viral diseases that have remained difficult to prevent with active immunization, and where suitable drug therapies are lacking (e.g. treatment of respiratory syncytial virus infection with palivizumab [35]).

The possible defense mechanisms triggered by passive immunization are manifold and, in essence, are defined by the interaction of the immunoglobulins (commonly IgG) with their binding part-

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ners: the targeted antigen, the complement system, and effector cells of the immune system.

The best characterized antiviral function of antibodies is neutralization of the virion by inhibition of attachment to or entry into the host cell [36]. Additionally, following opsonization of the virion, the Fc region of Abs (predominantly IgG and IgM) can recruit complement and immune effector cells bearing Fc receptors (FcR). The virion can thus be destroyed by complement dependent lysis (CDL), or antibody dependent phagocytosis (ADP). Furthermore, during productive infection viral envelope is present on the surface of infected cells, thus allowing antibody opsonization of these cells facilitating FcR and complement dependent effector functions (reviewed in [36]). Importantly these effector functions have proven essential in a number of antibody based cancer therapies [37,38], highlighting possibilities for similar interventions to deplete infected cells in chronic HIV infection. In fact it has been recently demonstrated that abolishment of the FcR affinity of a neutralizing antibody decreases its ability to protect against transmission of simian immunodeficiency virus–HIV chimera (SHIV) [39].

MECHANISMS OF ANTIBODY ACTIVITY IN HIV INFECTION

In principle anti-HIV antibody based therapies could comprise of two classes of antibodies: i) Virus specific mAbs which directly target the virion or infected cells to suppress infection and ii) mAbs directed towards cellular targets which either interfere with the infection process (e.g. anti-receptor Abs), or stimulate the innate or adaptive host anti-viral responses and thus indirectly confer anti-viral activity.

Viral Targets

HIV specific antibodies are detectable *in vivo* several weeks after infection [40]. The initial response is characterized by antibodies that bind the viral envelope proteins, but mostly do not neutralize the virus. Functional analysis of the early humoral response in the acute phase of HIV infection suggests that these antibodies, while non-neutralizing mediate anti-viral activity by eliciting effector functions *via* FcR and complement dependent mechanisms [40-42]. Over time antibodies with neutralizing capacity evolve in the majority of individuals, however due to HIV's high mutation rate it rapidly escapes this selection pressure, rendering the concurrent antibody response largely ineffective [1,43]. While humoral immunity overall fails to clear HIV during the natural course of infection, individual mAbs capable of neutralizing a broad range of viral strains have been isolated from patients [10,12,44]. Due to their exceptional breadth and potency these mAbs form the basis of current efforts to develop neutralizing antibody based vaccines. Likewise, they set the benchmark for inhibitor development that targets the viral entry process.

While the detail of molecular events leading to HIV neutralization are complex, and modes of action substantially differ between individual mAbs, the common principle of neutralization is shared amongst all. Antibody binding to the HIV envelope proteins prevents virus entry either by blocking the interaction with host cell receptors or subsequent steps interfering with the fusion process. Recent advances have highlighted the impact of neutralizing and non-neutralizing Abs on virus replication *via* recruitment of host effector functions, as described in Fig. (**1**). Cell-mediated virus inhibition (ADCVI) [45] is comprised of antibody-dependent cell-mediated-cytotoxicity (ADCC) [39], cellular-phagocytosis (ADCP) [46], stimulation of production of soluble antiviral factors and complement-mediated cytotoxicity (CDC) [41]). Considering the success of effector function based therapies in cancer treatment [37,38], development of HIV therapeutics that induce complement or effector cell lysis of virus or infected cells remains an intriguing strategy to explore.

In the absence of specific antibodies HIV activates the classical complement pathway *via* direct binding of C1q to gp41 [47], and the lectin pathway *via* attachment of mannose binding lectin to gp120 [48]. In addition antibody binding to the viral envelope efficiently enhance recruitment of the complement system. Yet, direct lysis of complement opsonized virions remains low as the virus counteracts complement activity by incorporating host cell derived complement control proteins which restrict complement action [49,50]. Nonetheless, although weak, antibody mediated complement lysis of HIV is detectable *in vivo*, and the ability of patient plasma to mediate lysis of autologous virus correlates inversely with viral load in acute HIV infection [41], suggesting that this effector mechanism may play a role in disease progression *in vivo*.

While current HIV therapies have tremendous success in limiting virus infection and spread, they fail to entirely eliminate both latently and productively infected cells. In our opinion the most valuable action conferred by antibody effector functions is their capacity to destroy infected cells, and thus eradicate the virus from the body [28]. During productive infection gp41 and gp120 are expressed on the surface of infected cells, thus antibody recognition of these proteins could initiate CDC, phagocytosis or ADCC. ADCC, in particular, is responsible for the therapeutic success of many monoclonal therapies in the treatment of tumors [51], and several studies have provided hints that this effector mechanism may also be of particular importance in HIV. Abs mediating ADCC of HIV infected target cells have been detected at virtually all stages of the disease [52], and recent reports on patient cohorts and animal studies suggest that the presence of ADCC mediating antibodies correlate with improved prognosis *in vivo* [53-55]. Additionally in an SHIV model the protective effect of an infused anti-HIV nAb has been determined to rely on the FcR stimulating ability of this mAb [39], implying that ADCC and/or phagocytosis mediate this protection.

It is important to highlight that non-neutralizing HIV-binding antibodies can also induce destruction of virions and infected cells. These antibodies, whilst failing to bind to the functional envelope trimer in such a way that prevents entry into host cells, nevertheless opsonize the virus. By recognizing a wider range of HIV envelope moieties (e.g. gp120/gp41 monomers, dimers, or gp41 stumps) this class of antibodies may more readily elicit effector functions. Bearing this in mind, should a therapeutic antibody be specifically designed to solely elicit effector functions or should it combine both neutralizing and effector activity? While the answer to this question in vaccine design is relatively straight forward - neutralization activity will definitely be required to block transmission - with therapeutic mAbs both possibilities can be imagined depending on the setting and application. In cases where the epitope of a non-neutralizing antibody is more frequently expressed on infected cells, or is more easily accessible than an epitope of a neutralizing antibody on a functional trimer, harnessing the non-neutralizing antibody to elicit ADCC or deliver an immunotoxin may be even more effective. In any case dosing and potential adverse effects must be considered carefully. Sub-neutralizing concentrations of neutralizing and non-neutralizing mAbs have been demonstrated to cause enhancement of HIV infection *in vitro* [56-60] (also reviewed in [36]), through either complement or FcR dependent uptake of opsonized virus [57]. Although antibody dependent enhancement (ADE) has been established as a mechanism of pathogenesis in other viral diseases, including Dengue virus [59], the role of ADE in HIV infection is controversially discussed in the literature [61]. In fact passive immunization with high doses of mAbs ineffective against the specific patient isolate did not lead to increases in viral load, indicating that ADE does not play a central role in HIV infection [20]. Nevertheless, the potential risk of infection enhancement *via* Fc or complement routes remains and must be carefully investigated and ruled out when antibody therapeutics are developed.

In addition to mAbs, other binding molecules engineered to interact with the virus and recombined with the Fc domains of Abs could be considered as antibody therapeutics. As an example fusion proteins composed of soluble CD4 (D1-D4 [62] or D1-D2 domains [63,64]) and human IgG-Fc have already been developed and probed clinically [65,66]. Additionally, binding to gp120 induces conformational changes in CD4, exposing a CD4 induced site (CD4i). Coupling CD4i domains to CD4-Fc reagents improves their neutralization potency by stabilizing gp120 binding [67]. These approaches, if built upon, would allow combination of target specificity with certain antibody effector functions or other modifications (e.g. immunotoxins) to neutralize virus and eliminate infected cells.

HOST TARGETS

Targeting Entry Receptors

A number of drugs targeting the cellular HIV receptors (CD4, and coreceptor CCR5 and CXCR4) have been developed over the years and CCR5 specific inhibitors in particular are nowadays an integral component of the therapeutic spectrum available to treat HIV infection [68]. Preceding the development of small molecule and peptide based inhibitors mAbs targeting CD4 and CCR5 were developed in the hope of generating suitable therapeutics [69,70]. Their principle mode of action is the same as that of virus directed antibodies: interference with viral entry. Notably, approaches that down modulate receptor expression, rather than block receptor function, have proven to be most effective in limiting the emergence of escape variants *in vitro* [71].

Probably the most compelling advantage of compounds targeting the cellular receptors of HIV is their broad activity against divergent strains. Since all HIV isolates, regardless of which genetic subtype, depend upon the same receptors for entry, inhibitors acting against the receptor, rather than the viral envelope, will be less affected by the high genetic variability of HIV.

Antibody products targeting CD4 and CCR5 have been pursued over recent years, thus far with only limited success compared to classical antiretroviral drug therapies [72,73].

Anti-CD4 receptor mAbs have been considered for both treatment of HIV and rheumatoid arthritis [74]. Most monoclonals targeting CD4 were found to be immunosuppressive as they blocked the CD4-MHC II interaction and hence were abandoned [75]. Ibalizumab (Taimed Biologics), a humanized mAb, targeting domain 2 of human CD4 does not prevent attachment to MHC II or gp120, yet blocks HIV entry [70] and showed notable *in vivo* activity [76]. However, a potential clinical application of the mAb is limited due to its extremely short half life (3.3 days [76]), necessitating the regular administration of exorbitantly high antibody doses. A major factor in the curtailed half life appears to be antigen-mediated clearance of the antibody through recycling of CD4 [77], which may represent an inherent limit to the half life achievable for CD4-directed mAbs.

As for CD4, targeting the coreceptors CCR5 and CXCR4, has the potential to provide broadly active inhibitor strategies, and both small molecule inhibitors and Abs targeting the coreceptors have been developed over the years [68,78]. CCR5 is the predominant co-receptor used by HIV during transmission and in earlier stages of the disease. Inhibition of CCR5 is thus of particular importance [79]. Importantly, natural occurring homozygocity for a 32 base pair deletion in the coding segment of CCR5 abolishes CCR5 expression and infers protection from infection by CCR5 utilizing HIV isolates (R5) and HIV infected individuals heterozygous for the deletion in CCR5 have a slower disease progression [80,81] (reviewed in [79]). Since individuals homozygous for this polymorphism appear healthy, CCR5 was recommended as a potential target for anti-HIV therapy [82,83]. Yet application of these inhibitors is restricted to individuals that carry solely CCR5 utilizing isolates, and treatment with CCR5 directed inhibitors has proven to give rise to the evolution of virus variants able to enter *via* alternate co-receptors [84] or able to enter *via* antagonist bound CCR5 [85,86].

It has been hypothesized that mAbs targeting the gp120 binding site of CCR5 could potentially provide a physically larger obstacle to the virus, and thus prevent or reduce the development of escape mutants able to utilize antagonist bound CCR5. Two anti-CCR5 mAbs have been developed and probed in clinical trials; HGS004 (Human Genome Sciences) [72] and PRO 140 (Progenics) [73]. While these trials underline the antiviral effect of CCR5 targeted antibody interventions, limitations of these approaches remain. Currently frequent high dose administration is required, and the consequences of continuous anti-receptor mAb treatment are not yet known.

Receptor targeting antibody therapies, while in principle attractive, are challenging to develop as many additional safety concerns arise. The mAbs' interaction with the receptor must be ascertained not to disturb natural function (activate cells, induce aberrant cycling, or recruit complement and effector cells leading to destruction of healthy cells). As a consequence these therapeutic mAbs are usually engineered as IgG2 or IgG4 subtypes as these have limited ability to recruit complement and FcR effectors.

Nevertheless the clinical impact of targeting host factors may not be adequately assessed in animal experiments, as was found in the Phase I trial of TGN1412; an anti-CD28 monoclonal that elicited extreme adverse effects in patients that were not predicted by preclinical animal experiments [87].

Alternate Host Cell Targets

In addition to the viral receptors, other cellular factors have been described to influence virus infectivity and interference with these factors may therefore allow modulation of HIV replication *in vivo*. For example binding of membrane expressed phospholipids on healthy host cells was found to reduce their infection by R5 isolates *via* triggering the release of chemokines CCL-3(MIP-1α) and CCL-4 (MIP-1β), ligands of CCR5 known to block R5 virus entry [88,89] highlighting yet another potential avenue for therapeutic antibody action *via* stimulation of innate defense mechanism against HIV.

Interaction between integrins incorporated into the viral particle and their cellular counterparts are known to influence HIV infectivity substantially [90-92]. Interference with the LFA-1/ICAM-1 interaction, an integral component of the virological synapse, leads to a 10-fold reduction in HIV infectivity *in vitro* [93-95]. Additionally rapid activation of LFA-1 results from direct interaction of HIV-1 gp120 with the gut homing receptor α4β7-integrin, facilitating efficient cell-to-cell spreading of HIV-1 [91]. However the function of integrins needs to be better understood before specific therapeutics can be developed. Interestingly, integrins have previously been investigated as drug targets to modulate the proliferation and migration of inflammatory and tumor cells [96,97]. Clinical use of two mAb integrin inhibitors, Natalizumab (Tysabri, BiogenIdec/Elan) [98] and Efalizumab (Raptiva, Genentech, Inc.) [99] have highlighted the potential, but also the risks, of integrin inhibitors. Following initial clinical success, both products were abandoned due to observed risk of progressive multifocal leukoencephalopathy (PML) [100-102].

Systemic administration of Abs for long-term treatment of chronic HIV infection or prophylaxis is unlikely to be pursued as to date these products cannot compete with the comparatively cheap small molecule antiretroviral drugs. However, were cheaper production methods established, and improved therapeutic profiles defined, this drug class may become useful in some particular settings, which we postulate upon later.

ISOLATION AND PRODUCTION OF ANTIBODIES

In 2008 it was estimated that 67% of people living with HIV worldwide reside in Sub Saharan Africa [103], demonstrative of the fact that HIV is a disease predominantly affecting low and middle income countries. In order to significantly impact the worldwide HIV epidemic any new therapy needs to be affordable and practical for use in resource poor settings. Whilst many therapeutic mAbs have been approved for clinical use, the associated production and dispensation costs remain high, thus wide spread application of mAbs is limited. For therapeutic mAbs to become a realistic treatment option for HIV outside of a few, very specific clinical settings, the costs of production will need to be dramatically reduced.

Over recent decades technologies for isolation and generation of monoclonal antibodies have evolved dramatically. Since generation of the first *in vitro* antibody producing cells by establishment of murine hybridomas [104], a number of technologies have emerged that are still widely used, including the generation of human EBV transformed B cells [105,106], human hybridomas [107,108], chimeric and humanized mAbs (to reduce mAb immunogenicity [109,110]), and human (single chain) mAbs from bacteriophage display libraries [111]. Most of the mAbs against HIV described to date have been isolated from the B cells of infected donors using either hybridoma technology (e.g. nAbs 2F5 (MPER specific) [2,112], 4E10 (MPER specific)[7], and 2G12 (gp120 glycan specific)[4]), EBV transformation [11,106,113] (e.g. neutralizing V3 loop mAb 447-52 [114]) or phage display (neutralizing CD4bs specific mAb b12 [3], D5 [8]).

The introduction of large segments of the human heavy and κ light chain loci into the germline of transgenic mice allows for the targeted generation of antigen specific human B cells [115,116] and has become widely used within the past decade with an increasing number of human Abs derived from transgenic mice in clinical development and in efficacy trials [117]. In recent years transgenic mice have also been employed to derive gp120 specific mAbs [118].

The development of effective, direct cloning strategies for both the heavy and light chains of human mAbs from single B cells [119-121] has launched a new era of antibody discovery and a number of HIV specific mAbs described in the recent literature have utilized these novel methods [10,12].

Whilst antibody isolation strategies have rapidly evolved in previous decades, the large scale production of human Abs for clinical purposes remains challenging. Production of fully processed human antibody molecules requires mammalian cell culture systems, rendering their production technically complex and expensive. Prokaryotic expression systems present no alternative as prokaryotes do not have the capacity to appropriately fold multisubunit antibody molecules and to introduce prost translational modifications such as complex N-linked glycosylations, which are required to minimize the immunogenicity of a foreign antibody, and to tailor the effector functionality and serum half life of the mAbs [122,123]. To date mAb genes are usually cloned from the antibody secreting cells (e.g. hybridoma) originally isolated, and re-expressed in cell types particularly suitable for fetal calf serum-free mass production (e.g. CHO, Chinese hamster ovary cells). Yet, whilst engineered cells secrete antibody at high yields and are considerably easier and cheaper to maintain than hybridomas, alternative, more cost effective, technologies are urgently required in order for production of mAbs to compete with the more economical small molecule anti-retrovirals.

Recently plant cell culture has been pursued as a comparatively cheap alternative to produce appropriately glycosylated mAbs, and plant derived antibody therapeutics for cancer are in pre-clinical development [124,125]. Proof of principle studies have demonstrated that HIV specific Abs could also be generated in plants. 2G12, a carbohydrate specific mAb targeting gp120, retained neutralizing activity upon isolation from transgenic Arabidopsis thaliana [126], tobacco [127,128] and maize [129]. In principle these studies are promising; however scale up of production and purification methods for plant derived biologicals need to be further developed before these strategies can become widely employed.

The use of transgenic animals for production is another emergent approach. Secretion of recombinant proteins, including mAbs, in the milk or urine of transgenic animals (e.g. goats, pigs, sheep, or cows) has been achieved yielding desired, high concentration of the mAbs [130,131]. While the initial experiments confirmed that transgenic large scale expression is technically possible, these approaches still have many obstacles to overcome. For one, generation of transgenic animals is immensely complex and time intensive. In addition strategies for scale up and safety reservations concerning zoonotic pathogens need to be addressed before transgenic animals may become a valid alternative.

IMPROVING THERAPEUTIC ANTIBODY ACTIVITY

Therapeutic mAbs have proven successful in diverse disease settings including cancer and autoimmune, inflammatory and infectious diseases. The rapid approval of more than 25 therapeutic mAbs over the past decade demonstrates their potential [132], but widespread use has also highlighted several shortcomings of the current technologies and functional limitations of the products. Therapeutic doses are often relatively quickly cleared from the circulation, and possess limited tissue accessibility, requiring frequent high-dose infusion. These complex proteins are not bioavailable following oral administration, and often unstable at room temperature. However, structure function analysis of antibodies over the past decade has allowed development of a number of engineering strategies which aim to overcome some of these limitations [122].

Steering Function

Affinity for the target epitope critically affects antibody function and can be successfully improved by affinity maturation of the antibodies' complementarity-determining regions (CDRs). This process is frequently employed to advance existing, licensed antibody therapeutics [133]. Alternatively, it has recently been observed, that engineering broadly neutralizing antibodies into stable dimers dramatically enhances their target affinity, and as a result neutralization potency [129,134].

The effector functions elicited by an antibody, and the potency of these functions, are largely determined by isotype. Upon identification of an antibody with desirable binding properties, the Fc region is often genetically engineered for production and an appropriate isotype is chosen for the indicated clinical application. Antibody therapeutics which aim to mediate destruction of targeted cells (e.g. most approved anti-tumor therapeutics) utilize IgG1 in order to harness its high affinity for FcRIII and Cq1 [135], and thus possess the ability to mediate ADCC, ADCP and CDC. While IgG3 also mediates these effector functions, the IgG3 hinge region is relatively susceptible to proteolysis, causing a decreased half life of IgG3 mAbs. As a result IgG3 mAbs are generally not considered advantageous for clinical application [136]. Elicitation of effector mechanisms, in particular ADCC, may be a very attractive therapeutic application for Abs against HIV, particularly in combination with supplementary strategies aiming to decrease the reservoir of infected cells in chronic infection. Yet, when considering FcR recruitment, the potential infection enhancing effects must be carefully ruled out in order to limit adverse effects [36].

Additionally, the FcR affinity of a given isotype can be modified *via* sequence editing in order to steer effector functions. Engineering reduced affinity for the inhibitory receptor FcRIIb, and increased affinity for ADCC mediating FcRIII, confers increased ability to mediate ADCC of target cells [137]. Likewise, improvement of Fc affinity for C1q infers improved ability to mediate

complement-dependent lysis of virions or infected cells [138]. Alternate approaches to improve complement lysis activity of HIV virions or infected cells rely on the addition of complement activating factors or biological agents that interfere with negative regulators of the complement pathway and thereby upregulate complement lysis activity [139,140].

The glycosylation pattern of an Fc domain is a key modulator of an antibody's ability to mediate effector functions and glycoengineering has introduced novel methods with which to direct antibody activity [141]. Notably, removal of fucose from antibody associated carbohydrates was found to dramatically enhance ADCC, highlighting a mechanism by which to specifically promote this effector function [142].

When targeting host proteins, elicitation of cytotoxic effector functions against healthy host cells must be carefully ruled out. Several licensed mAbs for which function is based on blocking a receptor molecule without affecting cell function and survival, have successfully employed isotypes IgG2 and IgG4 (e.g. Panitumumab, EGFR blocker; Natalizumab VLA-4 Integrin blocker [136]). However, for IgG4 isotypes Fab arm exchange with endogenous IgG4 has been described, which can substantially reduce *in vivo* activity [143]. This mechanism should be considered carefully when designing mAbs directed towards host cells as exchange between endogenous virus-directed IgG4 and therapeutic host-directed IgG4 could produce hybrid antibodies capable of recruiting virus directly to the host cell surface. This process is proposed to be responsible for the unexpected prevalence of JC virus infected oligodendrocytes in clinical trials with the IgG4 mAb natalizumab [143]. Stabilizing mutations may need to be introduced in future therapeutic IgG4 molecules to prevent Fab arm exchange [143]. Likewise, IgG2 molecules may require engineering of the hinge region to limit disulfide shuffling in serum, and reduce the *in vivo* production of structural isomers which allow hybridization between exogenous and endogenous IgG2 [144-146]

Recombinant polyclonal Abs are a new class of biopharmaceuticals for the treatment or prophylaxis of human diseases that aim to mimic the diversity and expediency of the immune system [147,148]. As polyclonal antibody responses elicited *in vivo* are directed at multiple epitopes individual antibodies are often able to bind the same antigen in close proximity allowing for optimized antiviral activity and recruitment of effector functions. A cocktail of recombinant polyclonal Abs may opsonize virions or infected cells in a similar fashion facilitating efficient virus neutralization, activation of complement and Fc receptor recruitment. Polyclonal antibody therapies have not yet been widely used, however two products are in development Sym001 (anti-Rhesus D), consisting of 25 different Abs, and Sym 004, a combination of two Abs directed against the epidermal growth factor receptor (EGFR) [149,150]. Polyclonal Abs could be particularly interesting in the context of infectious diseases as a polyclonal response will be more difficult for pathogens to evade than treatment with a single mAb. In addition, the recombinant polyclonal antibody technology could provide activity against a broader range of pathogen strains [147].

In recent years bispecific Abs engineered to bind two distinct antigens have increased in importance (reviewed in [122]). Bispecific Abs have found several different applications, one of the most successful being the recruitment and activation of immune effector cells. Bi-specific Abs that redirect T cells to the desired target cell through engagement of CD3 and a target cell specific antibody fragment have successfully been used in clinical applications to treat non-Hodgkin's lymphoma and malignancies [151,152]. Similar approaches have been considered for treatment of HIV infection. Along these lines, a bispecific antibody composed of a non-neutralizing antibody directed towards the gp41 immunodominant region and an anti-CD89 antibody was recently shown to mediate destruction of HIV by neutrophils [153].

Improving Half Life

One major drawback of most current antibody therapeutics is the need for relatively frequent, repeated parenteral administration as the half life of mAbs is commonly low (on average 1-22 days [154]). While improved modes of antibody delivery (discussed below) may overcome the necessity of parenteral administration, a prolonged half life would be beneficial in all settings as it would allow reduction of administered dose. PEGylation is amongst the most widely used engineering strategies to generate mAbs with increased *in vivo* longevity. Masking portions of mAbs by site-specific covalent attachment to polyethylene glycol can reduce immunogenicity and hinder proteolytic enzyme mediated degradation, and consequently improve half life [155]. Additionally PEGylation reduces renal clearance of smaller biopharmaceuticals, so may be of use in order to improve bioavailability of antibody fragments (discussed below). However, attachment of supplemental structures to mAbs may hinder epitope binding affinity and ability to mediate effector functions, so PEGylation sites must be chosen carefully, and biological function of modified mAbs ascertained.

The importance of the neonatal Fc receptor (FcRn) in regulating IgG homeostasis and maintaining serum IgG levels has only recently been understood [156-158]. Binding to FcRn rescues antibodies from degradation in the endosome, thus recycling them into the bloodstream. IgG bind in a pH dependent manner to FcRn upon acidification of the endosome, and are released from FcRn upon reaching the neutral extracellular environment. Engineering mAbs for improved FcRn interaction is therefore of particular interest in order to increase serum half life. Substitutions in Fc residues to improve FcRn binding at low pH, and encourage disassociation at neutral pH are a focus of antibody engineering [159,160] .

BUILDING UPON INTRINSIC ACTIVITY

Conjugating Antibodies

Immunoconjugates, mAbs engineered to possess additional cytotoxic functionality, have been successfully employed in other disease settings. Eliminating specific cell types could also prove advantageous if employed against HIV infected cells, particularly as a component of a strategy to eradicate (or diminish) the reservoir of infected cells [28,31]. An impressive variety of immunoconjugates including conjugation with cytokines, radioisotopes, toxins, drugs, enzymes or immunoliposomes have been developed for the treatment of a variety of diseases and are in clinical use [161] (Fig. **1**). All these approaches have one prerequisite in common; the targeting of the desired cell population must be highly specific to limit adverse effects.

Directing antiviral cytokines to infected cells by coupling of cytokines to mAbs (immunocytokines) may avoid the systemic toxicity associated with administration of cytokine alone, and also side-step the problem of rapid renal clearance of small proteins like cytokines. Immunocytokines which retain both cytokine potency and antigen binding affinity have been engineered, mainly *via* conjugation of cytokine to the carboxy terminus of constant regions, and, surprisingly, this conjugation does not necessarily block Fc mediated effector functions such as ADCC [162]. Chronic HCV infection is treated *via* administration of PEGylated interferon alpha (IFN-α) [163], however trials investigating the potential benefit of this treatment in HIV have not been encouraging, and it has been proposed that IFN-α may, in fact, participate in depletion of healthy CD4+ T cells [164]. The latter mechanism is dependent upon IFN-α stimulation of dendritic cells, thus may pose a particular problem for systemic administration of free interferon. Instead of systemic application of PEG—IFN use of immunoconjugated IFN-α, targeted directly to infected cells, may side-step this problem by inducing apoptosis specifically in HIV infected cells. In analogy to this, anti-CD20 IgG conjugated to four IFN-α molecules is being investigated for tumor therapy as preclinical tests suggested that

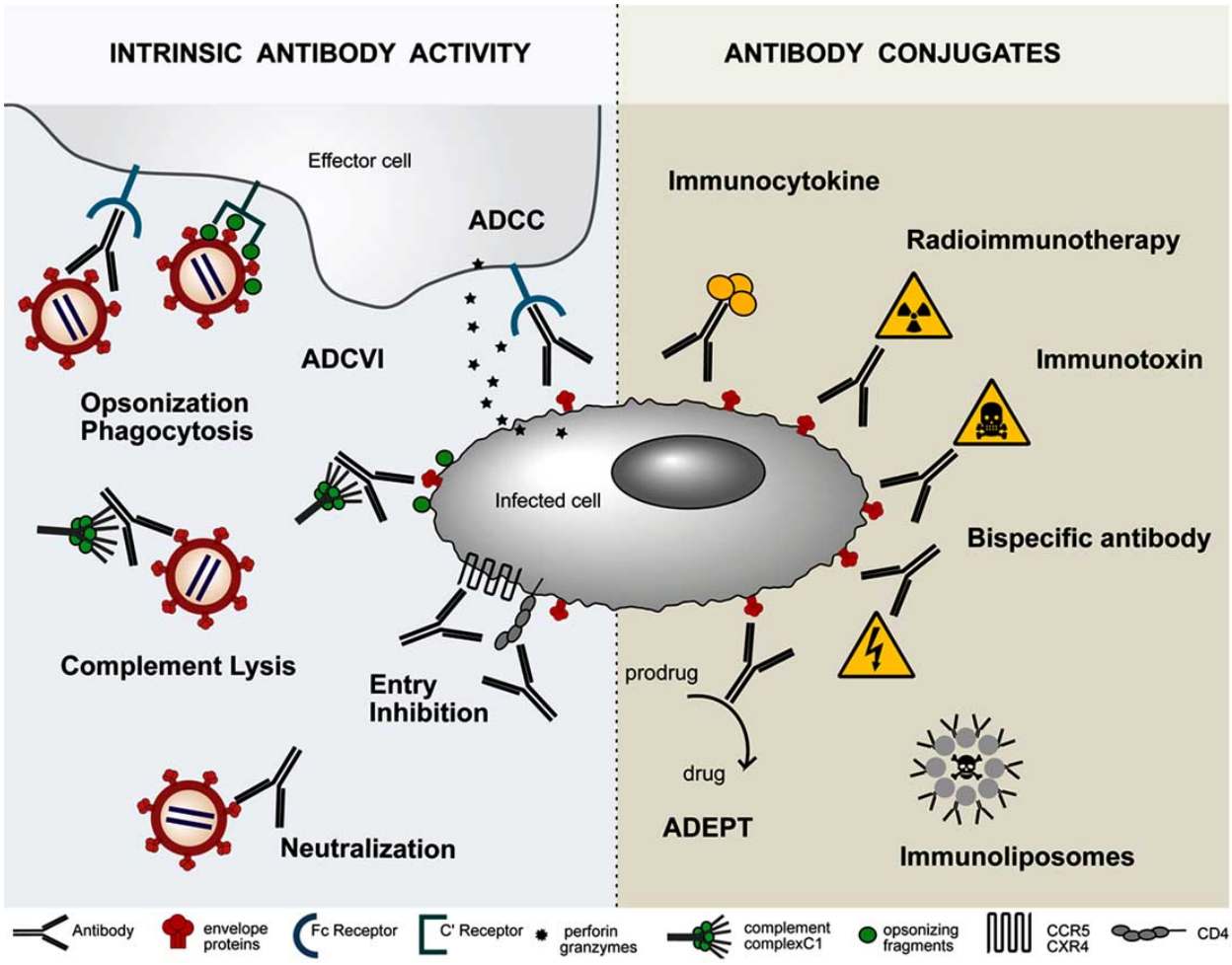

Fig. (1). Therapeutic antibodies in HIV infection.
Following opsonization of virions or virus infected cells, mAb can recruit FcR and C' mediated effector functions to neutralize or lyze virions or destroy infected cells, together termed ADCVI. Alternatively mAbs directed against entry receptors inhibit viral entry. mAb conjugation to cytokines, radioisotopes, toxins, drugs, or enzymes allows targeting of the conjugate activity directly towards infected cells.

this immunocytokine was able to mediate higher levels of ADCC and demonstrated superior therapeutic efficacy to mAb alone [165].

Radioimmunotherapy is successfully employed in cancer treatment and utilizes the antibody specificity to deliver lethal doses of radionuclides to the target cell [166]. Provided that target specificity is high, radioimmunconjugates directed to HIV envelope could be considered for depletion of HIV infected cells.

Immunotoxins are composed of an antibody (or antibody fragment) linked to a toxin, and have thus far mainly been probed in hematological malignancies and solid tumors (reviewed in [167]). Their compelling mode of action has recommended them for application in HIV infection [31]. For example ricin has been suggested for clinical use in HIV-1-infected patients to reduce or eliminate the viral reservoirs that remain after HAART [168]. Immunotoxins composed of gp41 specific Abs coupled to ricin A chain [169] or of gp120 specific Abs coupled to Pseudomonas exotoxin A [170] proved that killing of infected cells, through immunotoxins, is principally possible. However these constructs have not progressed to clinic and instead unraveled major shortcomings of this approach including nonspecific toxicity, immunogenicity, and instability of the compounds indicating that they would require substantial further development to overcome these drawbacks.

Antibody directed enzyme prodrug therapy (ADEPT) was developed to specifically deliver a drug activating enzyme to the tumor tissues *via* a tumor specific antibody. The subsequent systemic administration of a nontoxic prodrug leads to its conversion by the pre-targeted enzyme into an effective cytotoxic drug [171], and thus destruction of the target cell. Similarly antibody drug conjugates are designed to deliver a chemical compound to the target cells and release it after antibody internalization. This internalization directs the drug to endosomes, where fusion with activated lysosomes initiates metabolism and degradation of the contents, allowing release of the drug compound. A variety of cytotoxic drugs have been conjugated to mAbs (reviewed in [172], including small molecules that bind DNA (e.g. anthrazyklins [173]), alkylate DNA (duocarmycin [174] and calicheamicin [175,176]) and disrupt microtubules (e.g. auristatin [177] and cytotoxic antibiotic [178]). A comparable approach is employed by immunoliposomes. Antibody decorated liposomes containing small molecule inhibitors deliver drugs to specific cell subsets recognized by the antibody. This provides a prolonged therapeutic dose of drug

exclusively at the desired site of action, limiting adverse effects caused by activity on diverse cell types [179]. In principal these approaches can also be employed to deliver cytotoxic compounds to HIV infected cells using highly specific anti-envelope Abs.

Redesigning Antibodies

Antibody engineering has substantially evolved over recent decades and is not restricted to fine tuning full antibody molecules (discussed above), but has also explored a variety of antibody derivatives (e.g. antibody fragments, bispecific Abs), or categories of proteins that resemble antibodies in their binding properties. These strategies aim to derive molecules that can fulfill the most desirable functions of antibodies (e.g. binding specificity) while overcoming their drawbacks (e.g. cost of production).

Antibody fragments were the first derivatives investigated. Proteolytic digestion of Abs yield two fragments: the antigen binding variable fragment (Fv) and the crystallizable fragment (Fc). The latter is responsible for eliciting immune effector functions. Fv domains can be employed in the form of Fabs (paired heavy and light variable domains), scFvs (single chain variable fragments), or indeed antibodies lacking particular domains such as "miniaturized" or "domain" antibodies [180].

In principle antibody fragments possess several advantages over full antibody molecules. They are smaller in size and less complex in structure allowing replacement of costly mammalian cell culture production with production in prokaryotes [181].

In addition, antibody fragments are characterized by a higher stability which facilitates long term storage and potentially enhanced *in vivo* activity and can easily be engineered into multivalent structures (e.g. bi-specific compounds, immunoconjugates) [180]. Their reduced size may also be of an advantage in settings where tissue penetration of full mAbs is limited [182], or where accessibility to the antibody epitope underlies conformational constrains [183]. Their lower molecular weight allows higher dosing, yet at the same time renders the molecules more prone to rapid clearance. The latter is of particular concern and requires countermeasures (e.g. PEGylation) to maintain the half life of the fragments in a feasible range. The inability to mediate Fc-dependent effector functions, such as recruitment of complement or Fc bearing effector cells, can be a drawback, but as mentioned above may be overcome by utilizing the antibody fragments in form of immunoconjugates or as bi-specific molecules. Over the years a number of single chain Abs and Fab fragments against HIV have been described in the literature, most prominently the CD4 binding site specific b12 [184]. However most of these were less able to neutralize virus better as a fragment than as full antibody.

Camelids and Chondrichthyes produce antibody molecules entirely devoid of light chains, in which the antigen binding domain is formed by a single fragment termed VHH, or Nanobody® (reviewed in [185]). VHH, due to their advantageous characteristics (relatively small, stable molecules which can be produced relatively cost effectively), are considered attractive alternatives to mAbs. Llama VHHs have recently been successfully employed to select antivirals to HIV [186].

Non-immunoglobulin protein scaffolds have also been engineered to exhibit antigen specificities and affinities similar to that of antibodies [187]. For example, the designed Ankyrin repeat protein technology [188] has allowed selection of CD4 binding proteins which potently block HIV infection [189].

ANTIBODY ADMINISTRATION

Currently limitations in antibody delivery and manufacturing costs severely restrict the clinical application of antibody therapeutics. These factors are particularly problematic in HIV infection where both application of mAbs for prophylaxis and treatment of chronic infection would require long term administration.

Unlike small molecule antiretroviral drugs, complex proteins are not bioavailable following oral administration. Most currently approved therapeutic Abs are administered *via* injection or infusion requiring sterile conditions and trained staff (Fig. **2**). Formulating products that require less frequent administration, or are self administrable would certainly increase the accessibility of a given therapy. Several strategies have been explored over recent years, some of which have already entered the clinic.

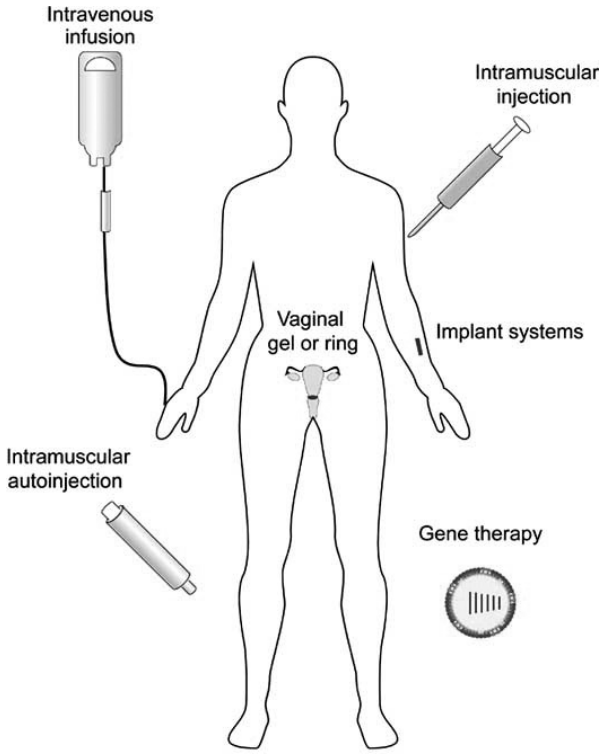

Fig. (2). Therapeutic Antibody Administration.
mAbs are traditionally administered *via* intravenous infusion, subcutaneous or intramuscular injection, or self-administered intramuscular injection with an auto-injector. Strategies to reduce administration frequency may include installing an antibody loaded implant, or using gene therapy to establish mAb production within the patient. If mAbs are to be prescribed prophylactically stable formulations administrable *via* microbicidal gel or vaginal ring would be advantageous in HIV.

Development of formulations allowing self-administration (e.g. autoinjector used for self-administration of Adalimumab [190]) may improve adherence. However, the bioavailability of mAbs and Fabs delivered by intramuscular injection remains relatively poor. Alternatively, upon appropriate formulation, therapeutic mAbs or smaller antibody fragments, may be systemically administrable *via* an implantable slab, as is commonly used in hormonal contraceptive therapy [191]. Eventually inhalation of antibody spray-dried into a micro particulate powder [192,193] may even be possible.

Production of controlled release formulations or implants dramatically reduce the frequency of administration required to maintain systemic therapeutic levels of antibody [194]. For example, encapsulation of the antibody into microspheres of biodegradable polymer results in slower release of bioactive antibody following IV, subcutaneous or intra-muscular injection (reviewed in [195]). The precise ratio of polymer to protein will need to be tailored to the specific features of each antibody in order to ensure retention of integrity, stability, and maintenance of affinity for all necessary

binding partners. Furthermore, for widespread use formulations that are stable over wide temperature ranges and allow for longer shelf lives would certainly be needed.

Gene therapy to induce therapeutic mAb production within the patient has recently been introduced as a potential alternative to passive infusion. Human stem cells, transduced with nAb genes, can be differentiated ex-vivo into antibody-secreting plasmablasts and plasma cells for transplantation back into patients [196], and initial attempts to express HIV specific mAbs in mice [197,198] and rhesus macaques [199] have proven that gene therapy approaches are in principle valid. The study by Johnson *et al*. was particularly promising as significant protection against SHIV was evident upon challenge [199].

While encouraging, these attempts are in early phases of development. The work of Johnson *et al*. has shown that immune responses directed against the expressed antibody therapeutics may be elicited. Depending on their nature, such responses could neutralize the expressed antibody, or even lead to severe adverse effects. Nevertheless, if proven safe, delivery of antibody therapeutics by gene therapy over prolonged periods is indeed an attractive alternative and is potentially useful for HIV prevention in high-risk populations and for treatment of chronic infection.

PROSPECTS FOR ANTIBODY THERAPY IN HIV INFECTION

As outlined throughout this review, possibilities for therapeutic antibody intervention in HIV infection exist in principal, particularly where advances in antibody engineering -proven successful in other therapeutic settings- are applied to HIV therapeutics.

Of several potential applications that may be considered classical passive immunization with a single mAb, at least in our opinion, has little chance to succeed, neither as prophylaxis nor as continuous treatment for HIV. In all settings where these potential therapies must compete with conventional small molecule antiretrovirals the cost of production, amplified by requirement for high dosing, parental administration, and limited *in vivo* activity due to rapid escape of HIV severely restrict possibilities of this treatment type.

A possible application for passive immunization could, however, remain in the form of short term treatment as salvage therapy. In cases where patients harbor multi-drug resistant strains of HIV, or when chronically infected patients must take “drug holidays” from HAART (e.g. due to complications arising from prescriptions for additional illnesses), therapeutic antibody cocktails may be of use. However, for a formulation to be widely applicable it must possess the ability to inhibit an extremely broad range of viral strains.

If mAb based therapeutics for HIV are to flourish they need to reach a wider audience, thus more practical and cost effective delivery modes are required. Controlled release options must be combined with antibody engineering to increase antibody efficacy, half life and effector functions in order to produce an effective therapeutic.

Considering the lack of an effective vaccine, using therapeutic Abs for prevention is, on a mechanistic basis, attractive. Currently this is not practicable, however, should gene therapy to express therapeutic Abs indeed prove safe and applicable in humans, expression of therapeutic Abs, for instance to prevent HIV transmission in high risk populations, may become possible. Another prophylactic approach, in which mAbs could potentially be explored, is as a component of microbicides. Encouragingly, a microbicide containing an antiretroviral drug has recently proven effective at preventing transmission in a large clinical trial [200]. However, besides high production costs, the relatively low stability of antibody preparations (both for storage and *in vivo*) is a further obstacle that needs to be overcome before application as microbicides can be considered. In order to provide longer-lived protection, topically active therapeutic biological gel formulations (reviewed in [201]), nanoparticulate delivery (reviewed in [202]), and locally insertable devices (vaginal rings, as used in contraception [203]) have been probed and may also serve to deliver therapeutic Abs.

The recent discovery of new, potently and broadly neutralizing mAbs may not only foster the development of vaccines that induce related responses, but may, in the shorter term, also allow development of antibody therapeutics based on these new monoclonals [10,12,204]. Using these highly specific Abs a new effort to create effective immunotoxic mAbs that induce increased killing of infected cells (through eliciting effector reactions or *via* various immunoconjugates, see Fig. **1**) may indeed be within reach, and could become particularly useful as a component of treatment strategies that aim to eradicate the reservoir of infected cells [28,31]. The latter is, in our opinion, the single most promising application for therapeutic antibodies, particularly when developed as component of a multi-faceted strategy to re-activate and purge the latently HIV infected cell reservoir.

Tremendous knowhow in antibody design for clinical applications has accrued over the past years. At the same time new mAbs to HIV with unrivaled potency have been defined. Applying the knowledge gained in antibody engineering in other therapeutic settings to put these new HIV specific mAbs to use is certainly worth attempting. Many hurdles still remain, and whether or not antibody based approaches will in the end prove successful in HIV infection remains to be seen. As we outlined in this review, theoretically a variety of application for therapeutic mAbs in HIV infection can be envisioned. Yet, without vast reductions in manufacturing costs mAbs will not provide a realistic treatment or prevention option for the majority of those infected with HIV worldwide. Still, the principal capacity of Abs to specifically target and destroy infected cells should be probed, as these approaches have the compelling potential to purge the reservoir of HIV infected cells.

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4. Results

4.1. Development of novel assay systems to assess cell-cell transmission and its neutralization

Development of novel assay systems to assess cell-cell transmission and its neutralization

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INTRODUCTION

HIV, as many other enveloped viruses, can efficiently and rapidly disseminate by directed cell-cell transmission (Sato, Orenstein et al. 1992; Jolly, Kashefi et al. 2004; Chen, Hubner et al. 2007). This transmission mode can occur by a variety of means and may involve both, cells that are productively infected (cis-infection) and cells that have trapped virus but remain uninfected (trans-infection (Granelli-Piperno, Delgado et al. 1998; Geijtenbeek, Kwon et al. 2000; Turville, Santos et al. 2004)). Depending on the cell type and the ratio of donor and target cells, transmission events were described to form a range of extracellular interaction structures, namely virological synapses (VS) (T-cell-T-cell (Jolly, Kashefi et al. 2004), Dendritic-T-cell (McDonald, Wu et al. 2003), Macrophage- T-cell (Groot, Welsch et al. 2008)), nanotubes (Sowinski, Jolly et al. 2008) or filopodia (Sherer, Lehmann et al. 2007).

As demonstrated for viruses from diverse families, dissemination through synapses allows viral spread in an environment largely excluded from the extracellular milieu and thus secluded from the humoral immune response (Law, Hollinshead et al. 2002; Favoreel, Van Minnebruggen et al. 2006; Timpe, Stamataki et al. 2008). It has been controversially discussed whether or not transmission of HIV-1 via the VS may be inhibited by neutralizing antibodies (NAbs), thus allowing evasion from the humoral immune response or therapeutic intervention (Jolly and Sattentau 2004; Sattentau 2010). However, recent studies have revealed, that the HIV-1 induced VS assembly is a highly ordered yet permeable structure (Felts, Narayan et al. 2010; Martin, Welsch et al. 2010). The activity of NAbs to protect against HIV infection has been well acknowledged in vivo (Trkola, Kuster et al. 2005; Hessell, Poignard et al. 2009; Mascola and Montefiori 2010), but whether direct virus neutralization or the inhibition of cell-associated viral spread are crucial for in vivo efficacy is still unknown. Currently the neutralizing activity of antibodies is investigated in assay formats that assess cell-free virus entry in a single round of infection (Montefiori 2005; Mann, Rusert et al. 2009; Montefiori 2009; Rusert, Mann et al. 2009). While these assays systems are very reliable and allow high-throughput analysis, they do not reveal whether the assessed neutralizing antibodies and entry inhibitors also block cell-cell transmission.

Distinct experimental approaches have been exploited to analyze cell-cell transmission and its neutralization. The most prominent being single virus tracking by confocal microscopy (Hubner, McNerney et al. 2009), assessment of target cell infection by intracellular p24 staining (Ganesh, Leung et al. 2004; Massanella, Puigdomenech et al. 2009) or fluorescent virus transfer (Chen, Hubner et al. 2007; Hubner, McNerney et al. 2009) by flow cytometry, or measuring de novo synthesized viral DNA by qPCR (Martin, Welsch et al. 2010). Even though these investigations have precisely measured target cell infection, discriminating between cell-free and cell-cell transmission remains difficult.

A number of attempts have been made to dissect these modes of transmission, eg disturbance of cell-cell contacts by keeping cultures in motion (Sourisseau, Sol-Foulon et al. 2007) and time course analysis of virus transmission to restrict analysis to a short time period during which cell-cell transmission occurs but cell free virus spread has not yet begun (Massanella, Puigdomenech et al. 2009; Martin, Welsch et al. 2010; Sigal, Kim et al. 2011). However, these assays require careful fine tuning of a relatively short infection interval, are labor intensive and may increase assay variability.

Thus, the primary intent of this study was to develop specifically tailored experiments strategies, which enable explicit separation of the two transmission routes and, in a further step, allow definitive analysis of cell-cell transmission neutralization. We devised two assay systems which allow unambiguous and quantitative assessment of cell-cell transmission.

RESULTS

Cell-cell transmission between PBMC and the reporter cell line TZM-bl is efficient and rapid

To assess whether HIV virological synapse-mediated dissemination is sensitive to entry inhibition by NAbs, we sought to develop a cell-cell transmission assay system that unambiguously differentiates between cell-associated and cell-free infection.

Based on the high stability and comparability of the widely used luciferase TZM-bl neutralization assay system (Montefiori 2005; Montefiori 2009), we developed a high-throughput cell-cell transmission assay by co-culturing this HeLa derived reporter cell-line with infected peripheral mononuclear cells ($PBMC^{HIV+}$) (Fig.1A). In order to dissect cell-cell transmission from cell-free infection during the $PBMC^{HIV+}$/TZM-bl co-culture, we made use of the fact that many CCR5 (R5) using HIV strains are only capable of efficiently infecting engineered, CCR5 and CD4 expressing TZM-bl target cell lines in the presence of polycations (Platt, Wehrly et al. 1998; Wei, Decker et al. 2002; Montefiori 2005). To mimick in vivo infected cells, PBMC were infected with the primary, replication competent JR-FL virus isolate (CCR5 using) at MOI 0.01 and four days later co-cultured with the adherent TZM-bl cells plated in presence of absence of the polycation DEAE-Dextran. Following 48h of co-culture, luciferase activity was quantified by luminescence readout (Fig.1A). Cell-cell virus transmission in the $PBMC^{HIV+}$/TZM-bl co-culture was polycation independent (Fig.1B). However, cell-free replication competent JR-FL infection was dramatically reduced in absence of DEAE Dextran (Fig.1C). Therefore, the omission of DEAE-Dextran as media supplement during co-culture reduces cell-free JR-FL infection to negligible levels.

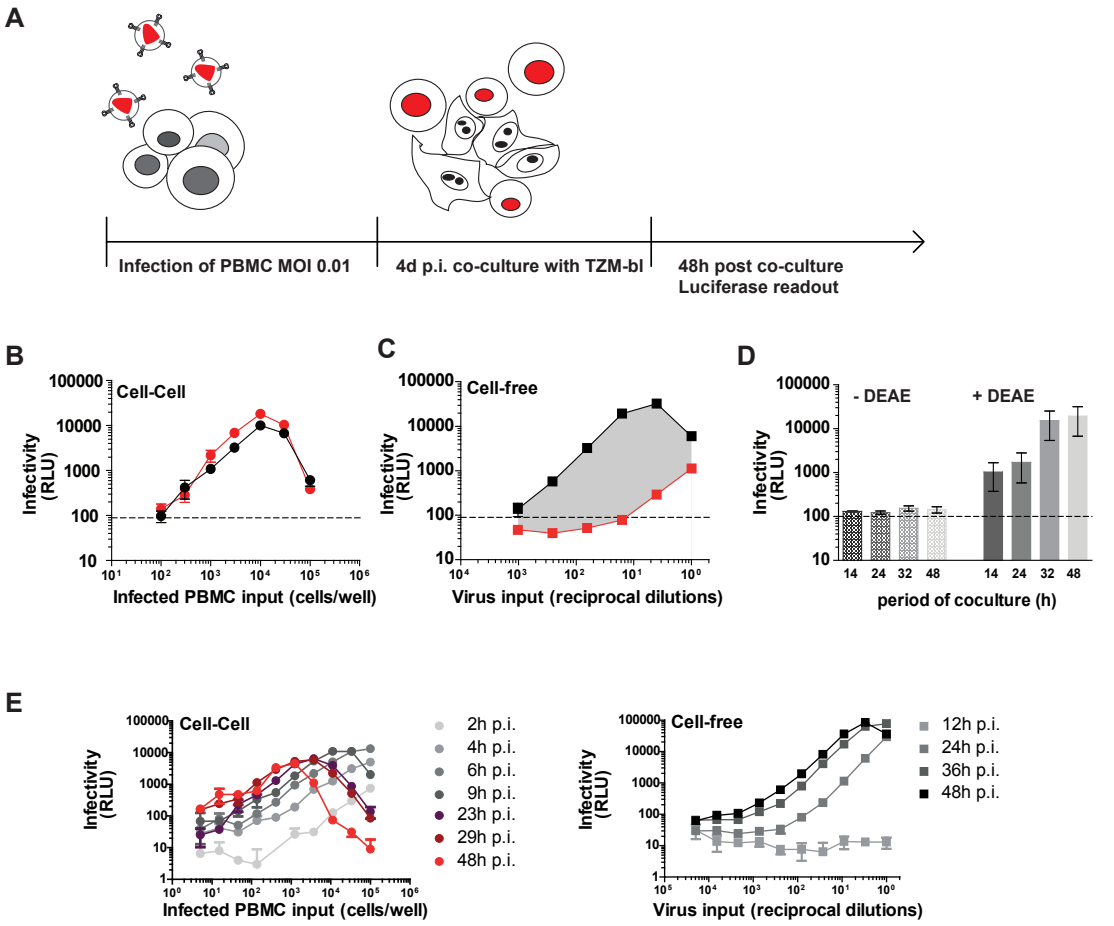

Figure 1 Cell-cell transmission between PBMC and the reporter cell line TZM-bl is efficient and rapid

(A) Scheme of the PBMC-TZM-bl assay set up. **(B) DEAE-Dextran is not required for effective cell-cell transmission of HIV-1$_{JR-FL}$ to TZM-bl cells.** Serial dilutions of JR-FL infected PBMC were incubated with TZM-bl cells in presence (black circles) or absence (red circles) of 10µg/ml DEAE-Dextran. Infectivity was measured by enzymatic activity of the luciferase reporter (relative light units (RLU)). Each infected cell input was probed in triplicate. Error bars represent SEM. One of four independent experiments is shown. **(C) Omission of DEAE-Dextran as media supplement abolishes cell-free JR-FL infection of TZM-bl cells.** Serial dilutions of cell-free JR-FL virus were used to infect the luciferase reporter cell line TZM-bl in presence (black squares) or absence (red squares) of 10µg/ml DEAE-Dextran. Infectivity was measured by induction of the luciferase reporter (relative light units (RLU)). Each virus dilution was probed in quadruplicates. Bars represent SEM. One of four independent experiments is shown. (**D) Newly produced virions during 48h do not interfere with the luciferase signal in absence of DEAE.** Serial dilutions of JR-FL infected PBMC were added on the unpermissive HeLa cell line. At the indicated time points, the supernatant was removed and added to the luciferase reporter cell line TZM-bl with or without 10µg/ml DEAE-Dextran. Infectivity was measured by enzymatic activity of the luciferase reporter (relative light units (RLU)) after 48h. Each virus dilution was probed in triplicates. Bars represent SEM. **(E) Cell-cell transmission is more rapid than cell-free transmission**. Cell-cell transmission of JR-FL from infected PBMC to TZM-bl in absence of DEAE Dextran (left panel) and cell-free JR-FL infection of TZM-bl in presence of 10µg/ml DEAE-Dextran (right panel) was monitored at the indicated time points by determining luciferase reporter production (RLU). Data points are means of triplicate measurements. Bars represent SEM.

In order to determine whether the increase in DEAE-Dextran independent cell-associated infection was caused by newly produced virions, we co-cultured the HIV-1JRFL infected PBMCs with HIV-1 unpermissive HeLa cells. At the indicated timepoints, the supernatant was harvested, centrifuged to remove all $PBMC^{HIV+}$ and consequently incubated with TZM-bl (with or without DEAE-Dextran) to assess for virus infectivity. No significant infection was measured in absence of DEAE Dextan, excluding an interference of the de novo produced virions during the $PBMC^{HIV+}$/TZM-bl co-culture (-DEAE) (Fig.1D). The reduction of infection was not due to ineffective virus production, as the produced virions were infectious in presence of DEAE (Fig.1D).
As already observed by other groups (Sato, Orenstein et al. 1992; Dimitrov, Willey et al. 1993; Chen, Hubner et al. 2007; Sourisseau, Sol-Foulon et al. 2007; Martin, Welsch et al. 2010), HIV cell-cell transmission proved to be accelerated in comparison to free virus infection, and induced luciferase production after only 4 to 6 hours as opposed to 24h in cell free infection (Fig.1E).

Only DEAE dependent viruses allow discrimination between cell-cell and cell-free transmission

It is known that polycations, including DEAE-dextran and Polybrene, substantially increase titers of retroviruses, including HIV-1 (Kahn, Lee et al. 1992; Davis, Morgan et al. 2002; Davis, Rosinski et al. 2004; Rusert, Mann et al. 2009). It is presumed that cell-free virus must overcome the electrostatic repulsion imposed by the negative charge on the cell surface in order to attach to its target receptors. Charged polymers such as DEAE Dextran are supposed not only to facilitate virus attachment via charge shielding (Davis, Rosinski et al. 2004), but also to stabilize post-attachment adsorption (Platt, Kozak et al. 2010). In order to assess cell-cell transmission in the $PBMC^{HIV+}$/TZM-bl system with a broader panel of virus isolates, we screened more virus isolates for DEAE dependence. The clear distinction between cell-cell and cell-free by omission of DEAE Dextran could not be applied to all CCR5 (R5) using viruses. Of the nine screened R5 using virus isolates only five genetically divergent viruses, the Tier-1 viruses ADA, the Tier-2 isolates YU-2, ZA015, ZA016 and the Tier-3 isolate ZA110 proved DEAE dependent. Similar to JR-FL they showed a decrease of cell-free infection (Fig. 2A) but no reduced transmission of cell-associated virus (Fig.3). In contrast, two Tier-1 isolates SF162, BAL and the Tier-2 isolate JR-CSF were DEAE independent and reached identical infectivity when cell-free infection was performed on TZM-bl cells without polycation (Fig 2A). A high positive amino acid charge of the V3 region has been associated with the SI phenotype and utilization of the CXCR4 coreceptor ((Fouchier, Groenink et al. 1992; Shioda, Levy et al. 1992; Cocchi, DeVico et al. 1996) reviewed in (Hartley, Klasse et al. 2005). As the tropism of the T-cell line–adapted SF162 can be changed from CCR5 to CXCR4

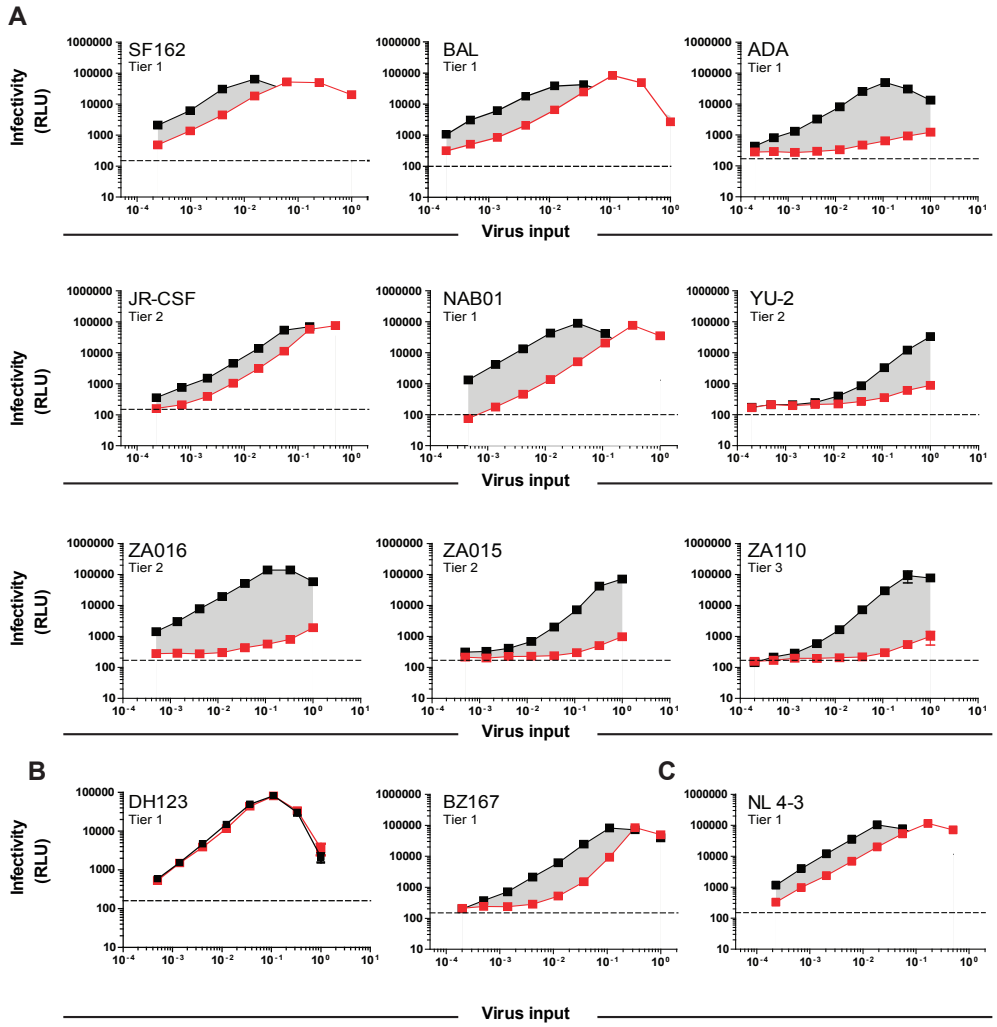

Figure 2 Only DEAE dependent viruses allow discrimination between cell-cell and cell-free transmission. (A) TZM-bl cells were infected with serial dilutions of cell-free R5 virus isolates in presence (black squares) or absence (red squares) of 10µg/ml DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each virus dilution was probed in quadruplicat. Bars represent SEM. One of two independent experiments is shown. (B) DEAE-Dextran independent cell-free infection of TZM-bl cells by certain X4R5 and (C) X4 using viruses. TZM-bl cells were infected with serial dilutions of cell-free R5 virus isolates JR-CSF and SF162, the R5X4 virus BZ167 and the X4 strain NL4-3 in presence (black squares) or absence (red squares) of DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each virus dilution was probed in triplicates. Bars represent SEM.

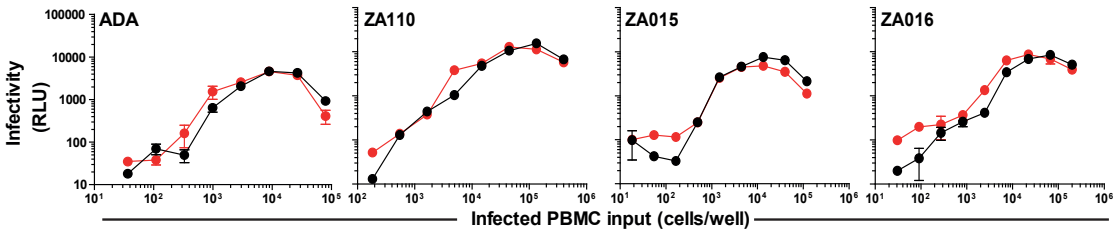

Figure 3 Absence of DEAE-Dextran as media supplement has no effect on cell-cell transmission of HIV-1 to TZM-bl cells. Serial dilutions of PBMC infected with different R5 isolates (ADA, ZA110, ZA015 and ZA016) were incubated with TZM-bl cells in presence (black circles) or absence (red circles) of DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each infected cell input was probed in triplicate. Error bars represent SEM. One of two independent experiments is shown.

by inducing only one amino acid substitution in the V3 sequence, which additionally increases the positive charge in the V3 loop (Harrowe and Cheng-Mayer 1995), DEAE independence is expected. Similarly, the CCR5/CXCR4 using strains DH123 and BZ167 (Fig.2B) and the CXCR4-using T-cell line–adapted virus NL4-3 (Fig.2C) were polycation independent. However, that the CCR5 users BAL and JR-CSF are DEAE independent is intriguing and the underlying mechanism of DEAE dependence needs to be further investigated. Thus, to assess cell-cell transmission with the $PBMC^{HIV+}$/TZM-bl assay system screening for DEAE dependency of all CCR5 using isolates is a necessary precondition.

Cell-free and cell-cell virus spread exhibit distinct and time-dependent sensitivity to entry inhibitors

Having established the TZM-bl based cell-cell assay system for replication competent R5 using HIV-1 strains, we next evaluated whether the mode of transmission had an influence on the potency of neutralizing antibodies and entry inhibitors. We compared the inhibitory potency of the CD4 directed DARPin 57.2 (Schweizer, Rusert et al. 2008), the gp120-directed tetrameric CD4-IgG2 molecule (PRO 542) (Gauduin, Allaway et al. 1996; Nagashima, Thompson et al. 2001) , the CD4bs NAb b12 (Barbas, Bjorling et al. 1992), the gp41-directed inhibitors (NAb 2F5 (Muster, Steindl et al. 1993) and the fusion inhibitor T-20 (Wild, Greenwell et al. 1993)). To accurately compare the inhibitory activity under cell-cell and cell-free virus transmission, we used the same virus stock to infect the PBMC and to tesT-cell-free inhibition. Importantly, the assay system was standardized to equal luminescence output in both approaches (10’000 RLU) and the readout was set 48h after co-culture.

We found thaT-cell-cell and cell-free virus transmission is blocked equally well by the CD4 directed DARPin 57.2 (Fig. 4A). However, HIV-1 envelope directed inhibitors showed a remarkably dichotomous pattern. The CD4bs specific NAb b12 and the tetrameric CD4-IgG2 molecule were significantly less effective in blocking cell-cell transmission (Fig. 4B). In contrast, the activity of gp41 MPER-specific NAb 2F5 and the fusion inhibitor T-20 were only marginally affected by the mode of virus transmission (Fig. 4C).

This disparity might be explained by the different stage of entry targeted by the investigated inhibitors. During cell-cell transmission, inhibitors targeting the cellular receptors have a clear advantage, as CD4 receptors are always accessible on targeT cells and inhibitors can bind immediately. In contrast, virus directed inhibitors depend on the initiation of the viral synapse formation and the ensuing accessibility of viral envelope proteins. As the viral synapse formation is initiated by gp120 binding to CD4 (McDonald, Wu et al. 2003; Jolly, Kashefi et al. 2004; Chen, Hubner

et al. 2007), CD4bs specific inhibitors are likely to only have a narrow time interval for action as evidenced by their loss of activity during cell-cell transmission. The MPER specific NAb 2F5 and gp41 directed the fusion inhibitor T-20 inhibit both virus transmission modes with equal potency suggesting that the time required for these inhibitors to act is similar during both entry processes. This is supported by the previous results reporting that MPER specific antibodies act preferentially in a cellular context following HIV envelope engagement by CD4. The MPER domain becomes more accessible, once gp120 has bound cellular receptors and envelope rearrangements have begun, allowing the antibodies to rapidly bind and neutralize the virus (Binley, Cayanan et al. 2003; Cardoso, Brunel et al. 2007; Frey, Peng et al. 2008; Alam, Morelli et al. 2009; Buzon, Natrajan et al. 2010; Frey, Chen et al. 2010).

Based on the previous observation that cell-cell transmission is rapid and efficient (Fig. 1E), we anticipated the readout to 6h after co-culture to investigate the neutralization potency during an early phase of cell-cell transmission. This change in co-culture time increased the previously observed considerable difference in neutralization sensitivity for both transmission modes. Cell -cell transmission resulted to be even more resistant to CD4bs inhibitors at 6h than at 48h. On the contrary, the activity of the inhibitor DARPin 57.2, the MPER specific NAb 2F5 and the fusion inhibitor T-20 were as potent in inhibiting cell-cell transmission as cell-free infection (Fig.4A,C). These intriguing results indicate that the inhibition of cell-cell transmission has a different kinetic depending on the targeted epitope and needs to be further investigated.

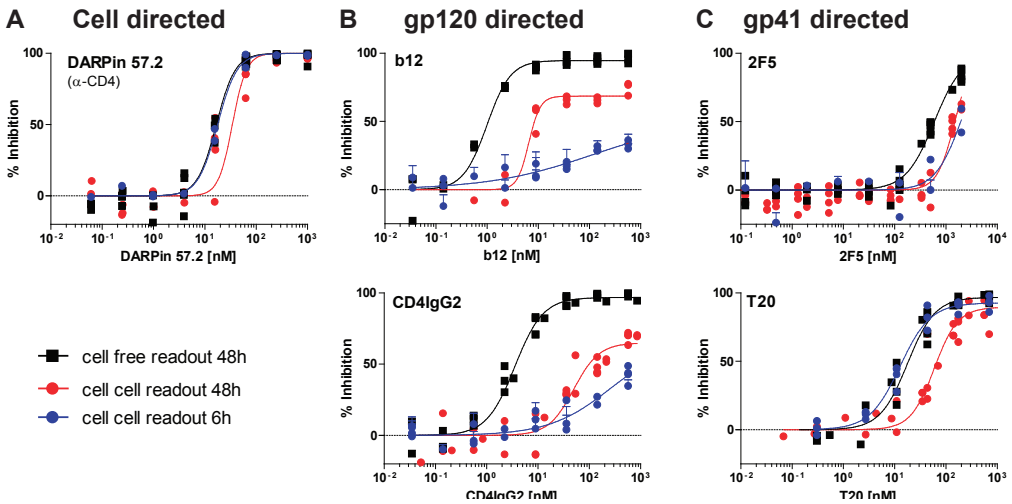

Figure 4 Markedly decreased sensitivity of HIV entry to gp120 directed inhibitors during cell-cell transmission.
(A-C) TZM-bl target cells were either infected with cell-free replication competent JR-FL (black squares, with DEAE) or cocultivated with JR-FL infected PBMC (red and blue circles; no DEAE) and inhibition by cell directed (A), gp120 directed (B) and gp41 directed (C) antibodies and inhibitor was studied. Infection was determined by measuring luciferase production at 48h for cell-free infection (black squares) and cell-cell transmission (red dots) or at 6h for cell-cell transmission (blue dots).

Assessment of cell-cell transmission inhibition requires the absolute distinction between cell-cell and cell-free transmission modes

The differences in the inhibition pattern were additionally probed with a broader panel of inhibitors and virus strains (See Chapter 4.2 “Cell-cell transmission enables HIV-1 to partially evade inhibition by potent CD4bs directed antibodies”).

Important for the validation of the differential inhibition patterns detected in the PBMC^{HIV+}/TZM-bl assay system was the investigation of whether the precise distinction between cell-free infection and cell-cell transmission had a significant influence on the resultant inhibitory pattern. We therefore compared the activity of DARPin 57.2, CD4-IgG2 and T-20 against the DEAE independent virus NL4-3 (Pandhare and Dash 2011) (Fig.5A) and the DEAE dependent virus JR-FL (Koyanagi, Miles et al. 1987) (Fig.5B). In an additional set up we co-cultured the JR-FL infected PBMC with DEAE pretreated TZM-bl targeT cells (Fig.5C), thus allowing any de novo produced virus to infect also in a cell-free mode (Fig.1D). Importantly, the addition of DEAE has been shown to have minor influences on assay sensitivity (Montefiori 2009; Rusert, Mann et al. 2009). The DEAE omission confirmed the observed differences in potency between the gp120 directed, the gp41 directed and the cell directed inhibitors against the DEAE dependent virus strain JR-FL (Fig.5B). However, when a DEAE independent virus, such as NL4-3, (Fig.5A) was assessed or cell-cell transmission of JR-FL was measured in presence of DEAE (Fig.5C), the CD4bs directed inhibitor CD4-IgG2 showed a different potency in inhibiting cell-cell transmission. This observation emphasizes the importance of a clear dissection between cell-cell and cell-free virus transmission. It also elucidates why in other assay systems, that do not distinguish between these transmission modes unambiguously, the difference in inhibitory activity may have been missed (Martin, Welsch et al. 2010).

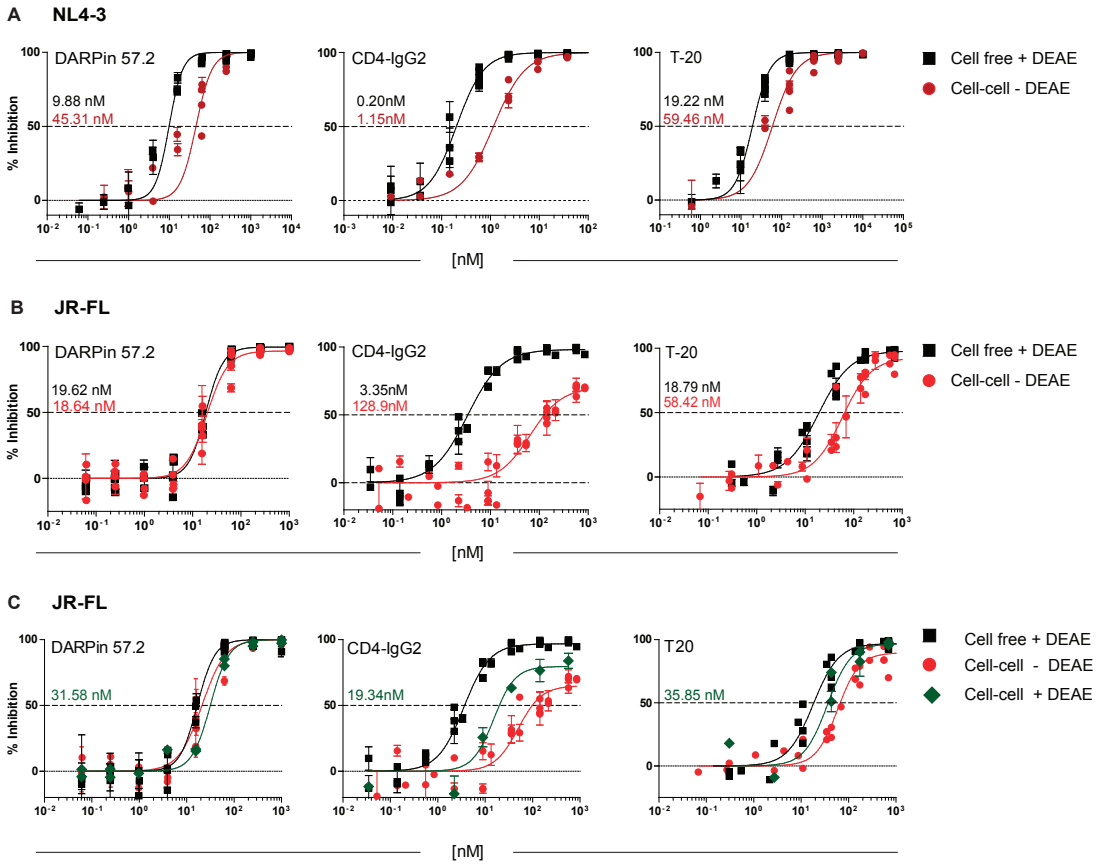

Figure 5. The unambiguous measurement of cell-cell transmission inhibition requires the clear distinction between cell-cell and cell-free transmission modes. (A) Inhibition of the X4 using strain NL4-3 and (B) R5 using strain JR-FL during cell-cell and cell-free transmission. TZM-bl target cells were either cocultivated with JR-FL infected PBMC (red circles, no DEAE) or cell-free virus (black squares, with 10µg/ml DEAE) in the presence of increasing doses of CD4-IgG2 (left panel), DARPin 57.2 (central panel) or T-20 (right panel). Infection was determined by measuring luciferase production after 48h and recorded as RLU. Red and black values denote IC50 (nM) of cell-cell and cell-free transmission, respectively. Data points represent means of duplicates from three independent inhibition experiments. Lines depict fitted dose response curves.

(C) The omission of the Polycation DEAE is crucial to dissect the two transmission modes. TZM-bl target cells were either cocultivated with JR-FL infected PBMC (red circles, no DEAE; dark green squares with 10µg/ml DEAE) or cell-free virus (black squares, with 10µg/ml DEAE) in the presence of increasing doses of CD4-IgG2 (left panel), DARPin 57.2 (central panel) or T-20 (right panel). Infection was determined by measuring luciferase production after 48h and recorded as RLU. Red and black values denote IC50 (nM) of during cell-cell and cell-free transmission, respectively. Data points represent means of duplicates from three independent inhibition experiments. Lines depict fitted dose response curves.

Establishment of a single round cell-cell transmission assay

We adapted the DEAE dependent TZM-bl infection assay system to assess cell-cell transmission of single-round replicating virus. Single-round replicating viruses were produced by cotransfecting an envelope deficient reporter virus construct carrying luciferase (pNL AM luc) or GFP (pNL gfp-AM) as a reporter and a plasmid encoding the envelope proteins (env) of the CCR5 using virus JR-FL. As already observed for replication competent viruses (Fig.1), the omission of DEAE reduced the infectivity of cell-free envelope pseudotyped JR-FL (Fig.6A). To assess cell-cell transmission of these env-pseudotyped viruses (ppHIV), we developed a new co-culture protocol (Fig. 6B). 293-T cells (10^4 per well) were seeded in 96-well plates, and transfected 24h after seeding. The transfected 293-T^{ppHIV+} cells were washed 24h post transfection and TZM-bl cells (10^4 per well, detached with 10mM EDTA) were added in the presence or absence of DEAE Dextran. After 48 hours of co-culture, infection of the TZM-bl cells was monitored by quantifying the production of the reporter luciferase. 293-T^{ppHIV+} cells transfected with pNL AM luc backbone produced a high amount of luciferase even in absence of an env (Fig.6C; left panel), thereby interfering with the target cell signal. Importantly, the use of the pNL gfp-AM backbone allowed for discrimination between donor and target cell infection, as the luciferase production was only induced upon infection in TZM-bl target cells (Fig.6C; right panel). The 293-T^{ppHIV+}/TZM-bl co-culture showed similar results as obtained in $PBMC^{HIV+}$/TZM-bl assay system. Cell-cell transmission was also predominantly polycation independent when assessing single round replicating viruses, and thus omission of polycations served as a suitable means to differentiate between the two modes of virus transmission. However, amongst the small screen of R5 using virus isolates we performed, no additional DEAE dependent R5 using pseudotyped isolate could be identified to assess cell-cell transmission with the 293-T^{ppHIV+}/TZM-bl assay system (Fig.7).

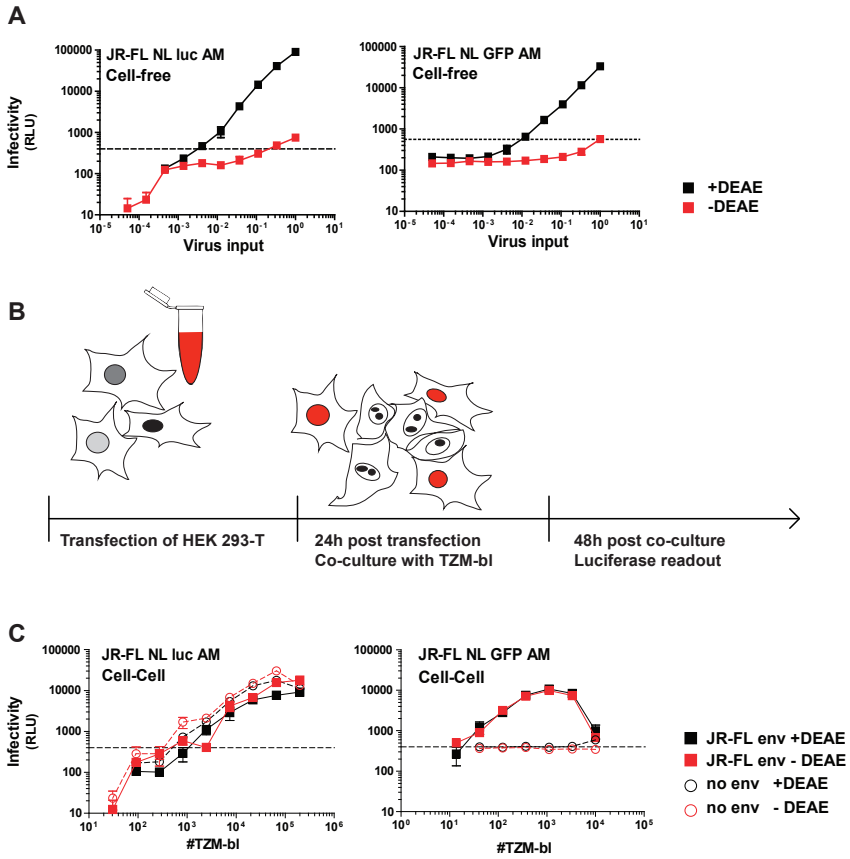

Figure 6 Omission of media DEAE-Dextran supplement abolishes single round cell-free JR-FL infection of TZM-bl cells. **(A) Cell free single round infection of TZM-bl target cells.** Serial dilutions of cell-free JR-FL virus were used to infect the luciferase reporter cell line TZM-bl in presence (black squares) or absence (red squares) of 10µg/ml DEAE-Dextran. Infectivity was measured by induction of the luciferase reporter (relative light units (RLU)). Each virus dilution was probed in quadruplicate. Bars represent SD. One of two independent experiments is shown. Omission of DEAE Dextran reduces cell-free viral infection to irrelevant levels.**(B) Scheme of the single round cell-cell transmission assay.(C) DEAE-Dextran is not required for effective cell-cell transmission of single round HIV-1$_{JR-FL}$ to TZM-bl cells.** Serial dilutions of JR-FL env (squares) and pNL luc AM (left panel) or pNL GFP AM right panel), or only backbone (open circles) transfected 293-T were incubated with TZM-bl cells in presence (black) or absence (red) of 10µg/ml DEAE-Dextran. Infectivity was measured by enzymatic activity of the luciferase reporter (relative light units (RLU)). Each infected cell input was probed in triplicate. Error bars represent SEM.

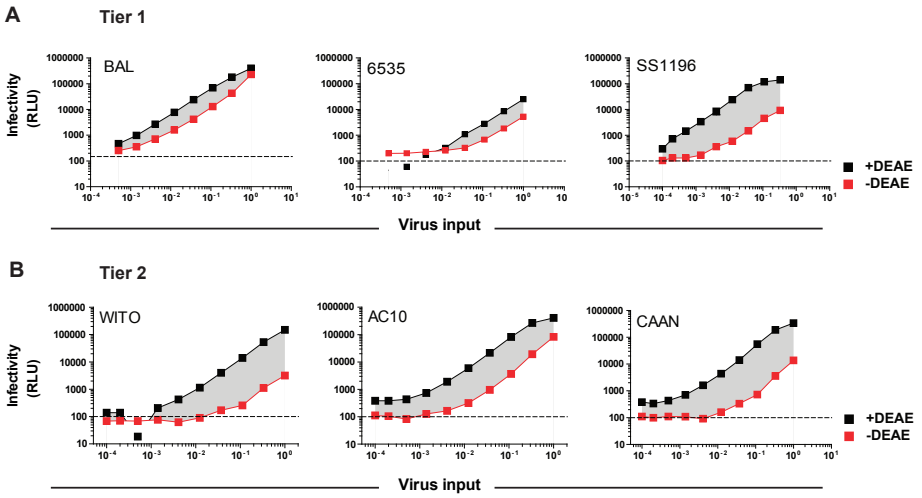

Figure 7 DEAE Dextran independent cell-free infection of TZM-bl cells by certain R5 viruses. DEAE Dextran dependent cell-free infection of TZM-bl cells by (A) Tier 1 and (B) Tier 2 R5 viruses. TZM-bl were infected with serial dilutrions of cell free pseudotyped R5 virus isolates in presence (black squares) or absence (red squares) of 10µg/ml DEAE Dextran. Infectivity was measured by induction of the luciferase reporter after 48h (relative light units (RLU)). Each virus dilution was probed in triplicate.One of two experiments is shown. Error bars represent SEM.

Restriction of cell-free infection by rhesusTRIM5α can be exploited to differentiate cell-cell and cell-free virus infection

Even though the PBMC^{HIV+}/TZM-bl and the 293-T^{ppHIV+}/TZM-bl assay systems clearly discriminate between cell-cell transmission and cell-free infection, they were inherently limiting and only allowed for the assessment of DEAE dependent virus strains. Hence, we sought to develop a further assay system that not only was independent of polycation usage, but also had a different mechanism of cell-free virus restriction. Rhesus macaque TRIM5α (rhTRIM5α), but not human TRIM5α (huTRIM5α), is known to severely restrict productive HIV infection (Sayah, Sokolskaja et al. 2004; Stremlau, Owens et al. 2004) at the post-entry and pre-integration steps by destabilizing the incoming viral capsid via ubiquitination (Chatterji, Bobardt et al. 2006; Stremlau, Perron et al. 2006). A recent publication by Pretel et al. revealed that TRIM5α supports innate immune signaling and acts as pattern recognition receptor (Pertel, Hausmann et al. 2011). However, cell-cell transmission is able to successfully overcome the restriction by rhTRIM5α (Richardson, Carroll et al. 2008).

To render the target cells resistant to cell-free infection, we adapted the published procedure of Richardson et al. by transducing selected target cells with virus like particles containing the insert of interest. VLPs were produced by co-transfecting 293-T cells with the packaging plasmid (pC-MVR8.91), the envelope VSV-G and the insert rhTRIM5α or huTRIM5α, which co-express GFP (Richardson, Carroll et al. 2008) (Fig. 8A). The produced VLPs were ultra-centrifuged and the viral particle content defined by p24 ELISA as already described (Rusert, Kuster et al. 2005). We transduced the A3.01-CCR5 T cell line (Chenine, Sattentau et al. 2000) by spinoculation (1200g 2h at 23°C) of 1000 VLP per cell (48 well plate; 50'000 cells/well) in presence of 10µg/ml DEAE. At 6 days post-transduction TRIM5α expression was examined by assessing GFP expression by flow cytometry. A transduction efficiency of at least 90% was reached in A3.01-CCR5^{rhTRIM5α} and A3.01-CCR5^{huTRIM5α} compared to A3.01-CCR5^{mock} transduced cells (Fig.8B). The transduction was stable during a period of 21 days (Fig.8C) and even after a freeze/thawing cycle of the transduced cells (results not shown).

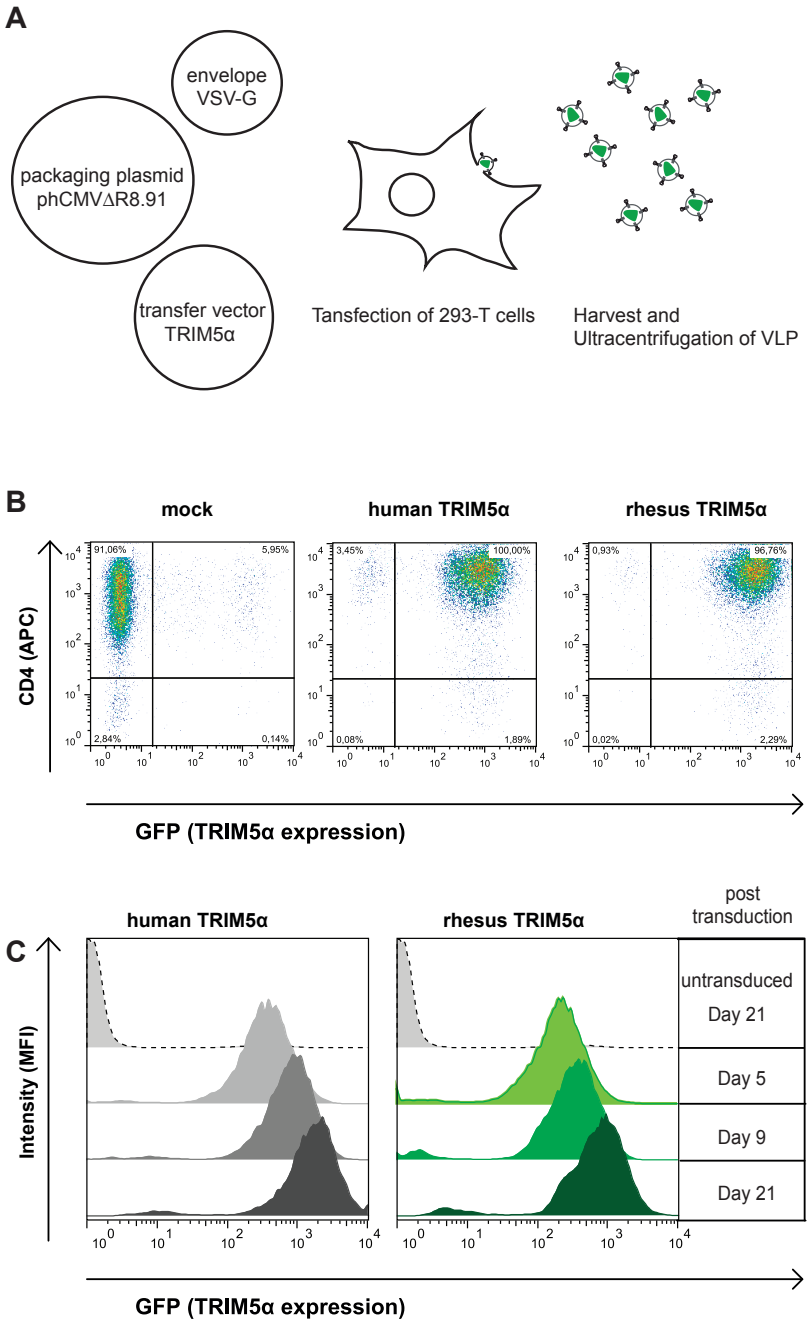

Figure 8 Transduction of T-cell line A3.01-CCR5 (A) Schematic of Virus like particles (VLP) production. (B) Transduction of the T-cell line A3.01-CCR5. Expression of human and rhesus TRIM5α is linked to GFP expression measured 6 days post transduction. One of five independent experiments is shown. **(C) The transient TRIM5α transduction is stable during 21 days.** Expression of human (left panel) and rhesus TRIM5α (right panel) measured at the indicated time points by flow cytometry. One of three independent experiments is shown.

Cell-cell transmission overcomes rhTRIM5α mediated restriction of HIV-1

Using this approach, resistance to HIV-1 infection could be conferred by rhTRIM5α expression in otherwise permissive cells. To confirm whether rhTRIM5α could protect this T-cell line from HIV-1 infection, we challenged the modified A3.01-CCR5^{rhTRIM5α} with either env-pseudotyped or replication-competent HIV-1. Resistance to single round virus infection was robust and resulted in close to 100% HIV-1 inhibition in rhTRIM5α transduced cells (Fig.9 A left panel). Upon challenge with replication competent HIV-1_{SF162}, 66% of mock transduced A3.01-CCR5 became infected, whereas HIV Gag expression remained at background levels in rhTRIM5α-expressing cells (Fig.9A; right panel). These data indicate that rhTRIM5α can restrict HIV-1 single-round challenge as well as in a multiple round infection and are consistent with previously published observations (Richardson, Carroll et al. 2008).

However, when infected A3.01-CCR5^{HIV+} cells were used as inoculum, A3.01-CCR5^{rhTRIM5α} cells became highly permissive to HIV-1 infection (30% positive cells, Fig.9B). The physical separation via Transwell chambers of A3.01-CCR5^{rhTRIM5α} and A3.01-CCR5^{HIV+} cells resulted in efficient restriction by rhTRIM5α, emphasizing the importance of cell-cell contact, as shown by Richardson et al. (Richardson, Carroll et al. 2008). It has been reported previously that cell-cell transmission increases infection kinetics and is more effective than equivalent cell-free infection, as it overcomes entry restricting obstacles, eg extracellular fluid phase diffusion and dissociation from the target cell (Sato, Orenstein et al. 1992; Dimitrov, Willey et al. 1993; Chen, Hubner et al. 2007; Sourisseau, Sol-Foulon et al. 2007; Martin, Welsch et al. 2010; Mazurov, Ilinskaya et al. 2010; Platt, Kozak et al. 2010). To investigate whether mere membrane proximity overcomes the entry restriction, we mimicked an enclosed virus-cell contact by enforcing attachment of cell-free replication competent HIV-1SF162 onto the target cells. We adsorbed HIV-1_{SF162} by spinoculation (2h at 1200g at 23°C) (O'Doherty, Swiggard et al. 2000) or by magnetic methods (Haim, Steiner et al. 2005). The comparison revealed that although the enforced adsorption increased infectivity, rhTRIM5α maintained efficient entry restriction of cell-free HIV-1_{SF162} (Fig.9C). These results confirmed that the increased transmission efficiency detected in our assay system was a result of direct a cell-cell interaction and not mere membrane proximity.

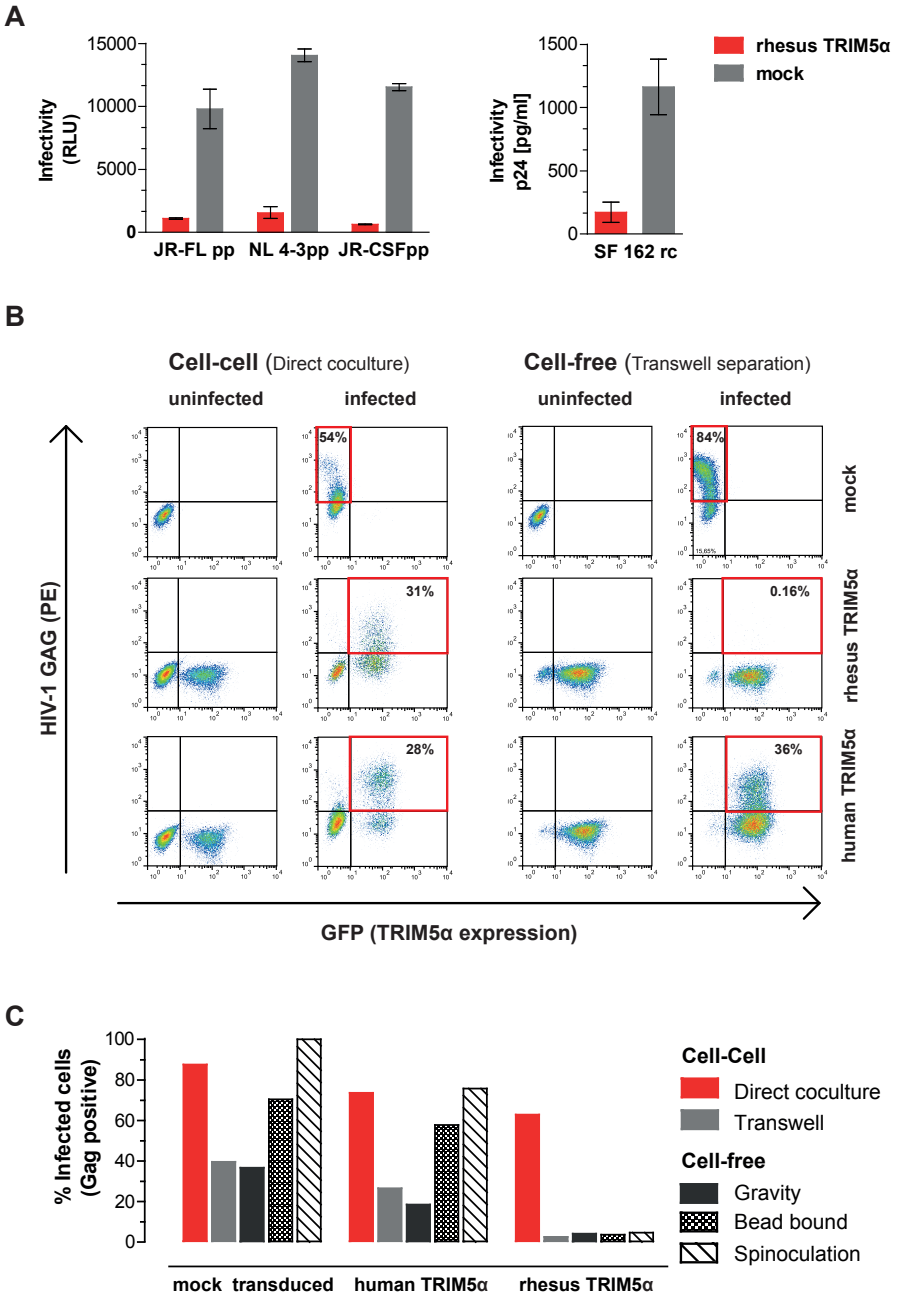

Figure 9 Cell-cell transmission overcomes rhTRIM5α mediated restriction of HIV-1 (A) Rhesus TRIM5α transduced cells are highly resistant to cell-free single round and multiple round infection. Infection of rhesusTRIM5α or mock transduced A3.01-CCR5 cells with the indicated env-pseudotyped, luciferase reporter viruses (left panel) or replication competent SF162 isolate (right panel). Infection of the reporter virus was determined by measuring luciferase production after 48h (recorded as RLU/ml). Infection of SF162 was monitored by determining p24 antigen production. Both cell free infection with single round, env pseudotyped virus and replication competent virus isolates proved to be almost completely restricted in rhTRIM5α transduced A3.01-CCR5 cells. One of two independent experiments for each virus isolate is shown. Error bars represent SEM. **(B) Cell-cell transmission overcomes rhTRIM5α mediated restriction of HIV-1.** Uninfected or SF162-infected A3.01-CCR5 cells (donors) were co-cultivated with the indicated A3.01-CCR5 target cells (mock treated (no gfp), rhTRIM5α (gfp positive), huTRIM5α (gfp positive)) either in direct co-culture (left panel or separated by transwells (right panel). Infection was assessed by intracellular HIV-1 Gag staining after 6 days of co-culture. Data show one representative out of three independent experiments. **(C) Cell-cell transmission but not enforced contact between virus and target cell overcomes rhTRIM5α mediated entry restriction.** Comparison of the infectivity of cell-free SF162 infection of i) spinoculated, ii) magnetic bead bound virus and iii) virus added without enforced adsorption with cell-cell transmission (direct cocultivation and transwell). Infection of mock treated, rhTRIM5α and huTRIM5α A3.01-CCR5 target cells was investigated. One representative out of three independent experiments is depicted. To allow comparison, data are normalized to infection levels obtained by spinoculating cell-free SF162 onto mock transduced cells.

High throughput luciferase assay adapted to assess DEAE independent viruses

We next sought to adapt the FACS based rhTRIM5α restriction T-cell to T-cell assay to a high-throughput assay with a luciferase reporter readout. We again used the reporter cell line TZM-bl to this end and transduced it with the rhTRIM5α vector. The transduction efficiency of the reporter cell line TZM-bl was dependent on the VLP input and was saturated with approximately 2000 VLP/cell (Fig. 10A). After transduction TZM-bl^{rhTRIM5α} were sorted to increase the GFP positive cells to a >90% pure cell population. As expected, the sorted TZM-bl^{rhTRIM5α} cell line was resistant to cell-free infection by single round infecting viruses (Fig.10B). Moreover, cell-free infection with replication competent DEAE independent virus isolates SF162 (CCR5), JR-CSF (CCR5), and NL4-3 (CXCR4) was clearly restricted in TZM-bl^{rhTRIM5α} (Fig.10C red squares). The co-culture of infected PBMC as infectious inoculums confirmed that multiple round cell-cell transmission was able to successfully overcome the entry restriction by rhTRIM5α (Fig.10D).

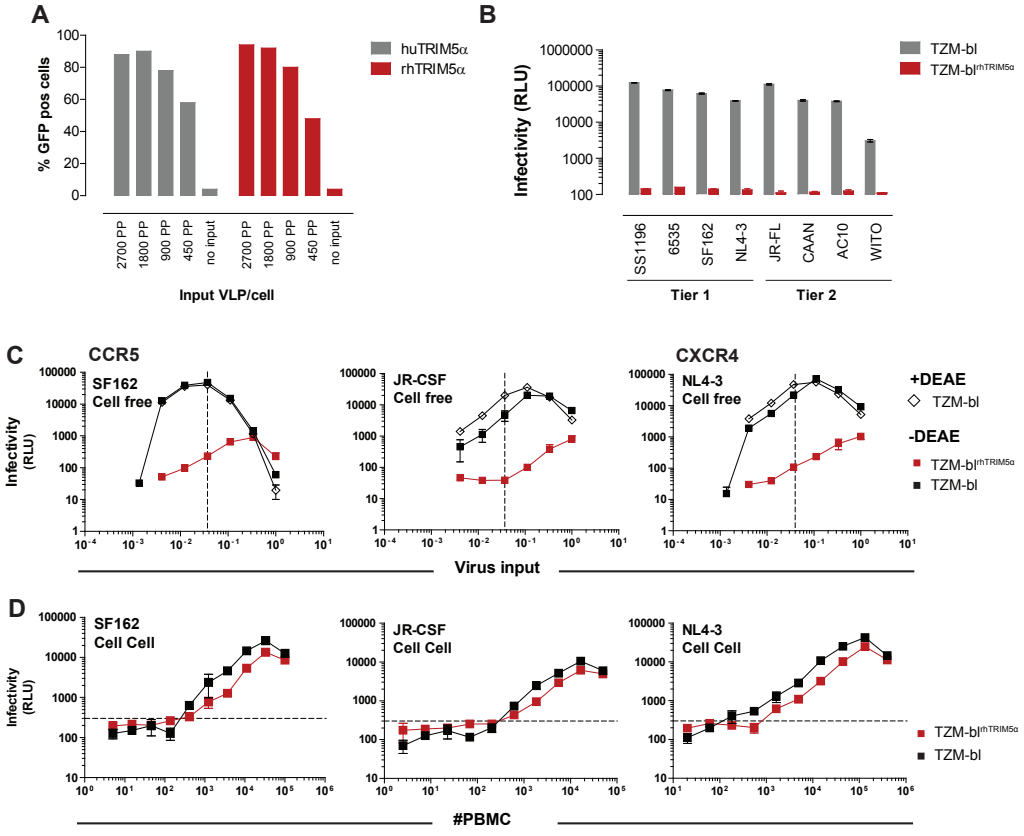

Figure 10 (A) Transduction of the reporter cell line TZM-bl. Expression of human and rhesus TRIM5α measured 6 days post transduction by FACS.**(B) RhesusTRIM5α transduced TZM-bl cells are highly resistant to cell-free single round infection**. Infection of TZM-bl^{rhesusTRIM5α} (red bars) or TZM-bl^{mock} (grey bars) cells with the indicated env-pseudotyped, luciferase reporter viruses. Infectivity was determined by measuring luciferase production after 48h (RLU). One of two independent experiments for each virus isolate is shown. Error bars represent SEM. **(C)TZM-bl^{rhesusTRIM5α} are highly resistant to cell-free multiple round infection.** Infection of TZM-bl^{rhesusTRIM5α} or TZM-bl^{mock} cells (in presence or absence of 10µg/ml DEAE Dextran) with the replication competent SF162, JR-CSF,and NL4-3. Infection was determined by measuring luciferase production after 48h (RLU). One of two independent experiments for each virus isolate is shown. Error bars represent SEM.**(D) Cell-cell transmission overcomes the restriction of rhesus TRIM5α**. Serial dilutions of SF162, JR-CSF, and NL4-3 infected PBMC were incubated with TZM-bl^{rhesusTRIM5α} (red squares) or TZM-bl^{mock} (black squares).Infectivity was measured by enzymatic activity of the luciferase reporter (relative light units (RLU)). Error bars represent SEM. One of two independent experiments is shown.

We adapted the PBMC^{HIV+}/TZM-bl$^{rhTRIM5\alpha}$ co-culture to single round virus by using the same co-culture protocol as described for 293-T^{HIV+}/ TZM-bl (Fig. 6). With the 293-T^{HIV+}/ TZM-bl$^{rhTRIM5\alpha}$ co-culture we were able to assess cell-cell transmission of DEAE independent pseudotyped viruses (Fig. 11B), without interference of cell-free infection (Fig. 11A).

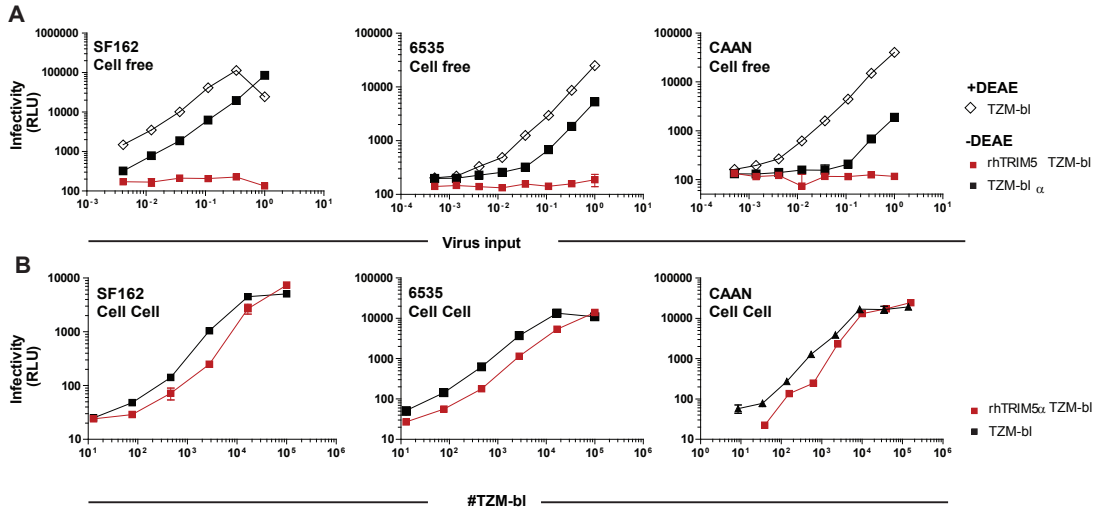

Figure 11 High throughput luciferase assay adapted to assess DEAE independent pseudotyped viruses. (A) Rhesus TRIM5α transduced TZM-bl cells are highly resistant to cell-free single round infection. Infection of TZM-bl$^{rhesusTRIM5\alpha}$ (red squares) or TZM-bl^{mock} (black squares without DEAE, open black squares with DEAE) cells with the pseudotyped SF162, 6535, and NL4-3. Infection was determined by measuring luciferase production after 48h (RLU). One of two independent experiments for each virus isolate is shown. Error bars represent SEM. **(B) Cell-cell transmission overcomes the restriction of rhesus TRIM5α.**TZM-bl$^{rhesusTRIM5\alpha}$ (red squares) or TZM-b^{lmock} (black squares) target cells were incubated with serial dilutions of transfected 293-T cells (envelopes: SF162, 6535, and NL4-3). Infectivity was measured by enzymatic activity of the luciferase reporter (relative light units (RLU)). Each infected cell input was probed in triplicate. Error bars represent SEM. One of two independent experiments is shown.

Establishment of rhesusTRIM5α transduction into peripheral blood mononuclear cells

An important concern was whether the rhTRIM5α based assays could also be adapted for primary cells. Freshly isolated and CD8 depleted PBMC obtained from three healthy donors were transduced one day after isolation (Fig.12A) and gene transfer efficiency was determined by analyzing the GFP expression at the indicated time points (Fig. 12B). One day after transduction 18% GFP positive PBMC^{rhTRIM5α} were detected, increasing up to 40% at day eight after transduction (Fig. 12B). As the PBMC needed to remain highly viable throughout the duration of infection experiments, the transduction efficiency was assessed in the subsequent experiments at day 4 post transduction. A maximum of 30% GFP positive, viable cells was reached with approximately 600VLP/cell (Fig. 12B). Addition of the polycation Polybrene (8µg/ml) and spinoculation (2h at 1200g at 23°C) resulted in superior transduction efficiency compared to the gravity sedimentation (Fig.12C; left panel). We explored the possibility of using a modified stimulation method to increase the PBMC^{rhTRIM5α} population, ensure its cell-associated infection and the cell-free infection of PBMCmock cells. We tested different co-stimulation protocols either only IL-2 Medium, anti-CD3 antibody coated plates with IL-2 Medium and co-stimulation on anti-CD3/anti-CD28 antibody coated plates with IL-2 Medium. Among these transduction conditions, stimulation with anti-CD3/anti-CD28 antibody directly after isolation and prolonged co-stimulation during the transduction period of four days exhibited the highest gene transfer efficiencies (Fig.12C; right panel). Having established the transduction procedure to yield maximal transduction efficiencies, PBMC^{rhTRIM5α} were sorted four days after transduction. A population of >90% transduced yet permissive cells were used in the following infection and co-culture assays (Fig. 12D).

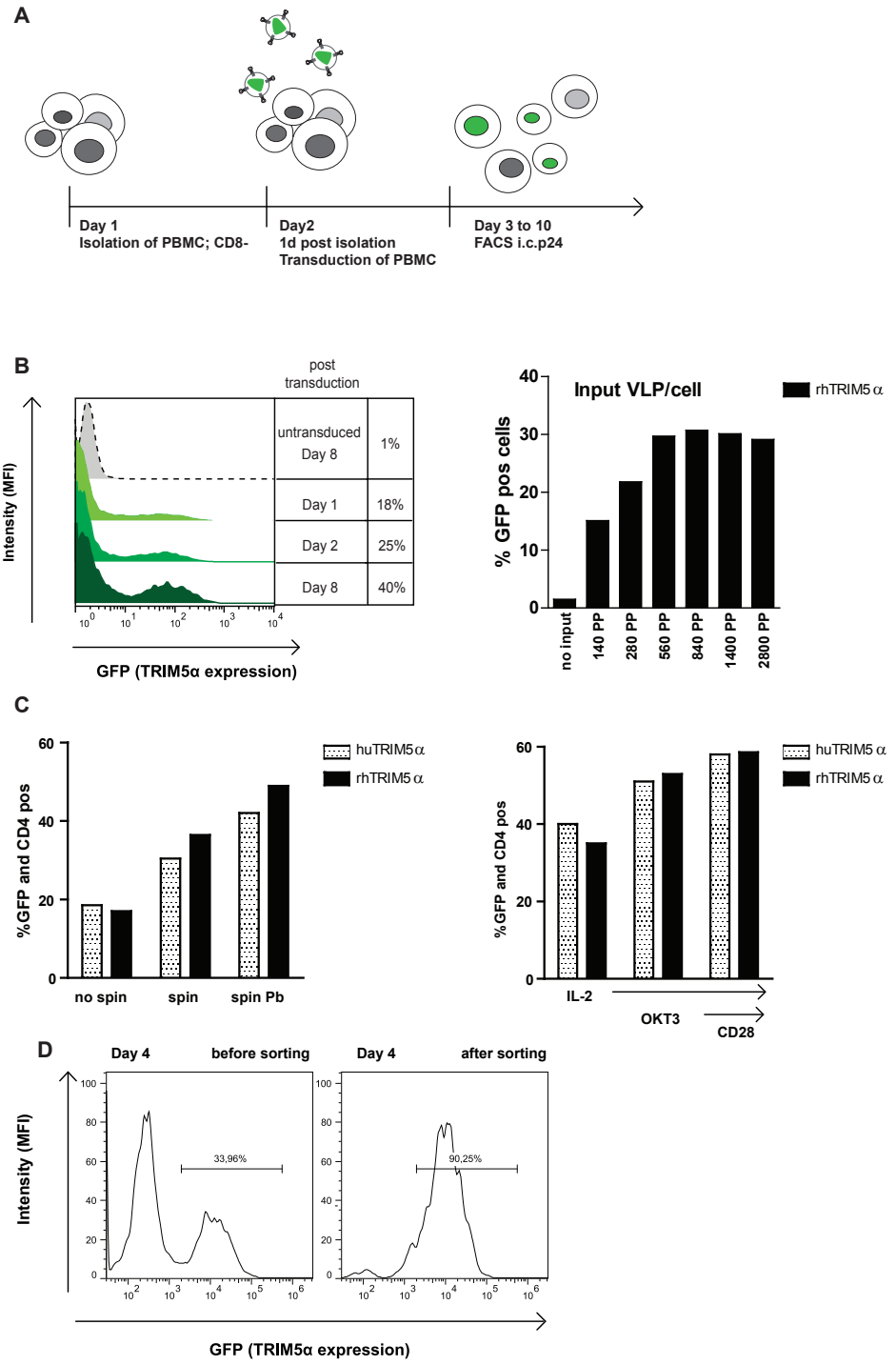

Figure 12 Transduction of primary cells with rhTRIM5α (A) Scheme for transduction procedure of primary cells. (B) Transduction efficiency of PBMC. GFP expression assessed at the indicated time points post transduction by FACS (left panel). VLP dependent GFP expression assessed at day 4 (right panel) **(C) Different transduction and stimulation methods to increase transduction efficiency of PBMC.** GFP expression was assessed 6d post transduction by FACS. Different transduction protocols (left panel) and stimulation methods (right panel).(D) Sorting efficiency of GFP positive PBMC at day 4 post transduction.

Cell-cell transmission overcomes restriction by rhesusTRIM5α in human PBMC

Following optimization of the transduction protocol, we subsequently investigated whether the cell-cell transmission could also overcome in primary cells restriction of cell-free HIV-1 infection by rhTRIM5α. We performed the infection and co-culture experiments with a method comparable to that employed in the A3.01-CCR5^{rhTRIM5α} assays (Fig.13A). After sorting on day 6, equal numbers of modified rhTRIM5α or mock transduced PBMC and unmodified, HIV-1 infected donor cells were mixed at a 1:1 ratio and co-cultured for 48hours. At day 7 after isolation, infection of the target cells was assessed by measuring the intracellular p24 expression by flow cytometry (Fig.13A). Co-culture of PBMC^{HIV+} and PBMC^{rhTRIM5α} resulted in 9% infected rhTRIM5α and 18% of mock transduced PBMC, whereas no cell-free infection of the PBMC ^{rhTRIM5α} could be observed.

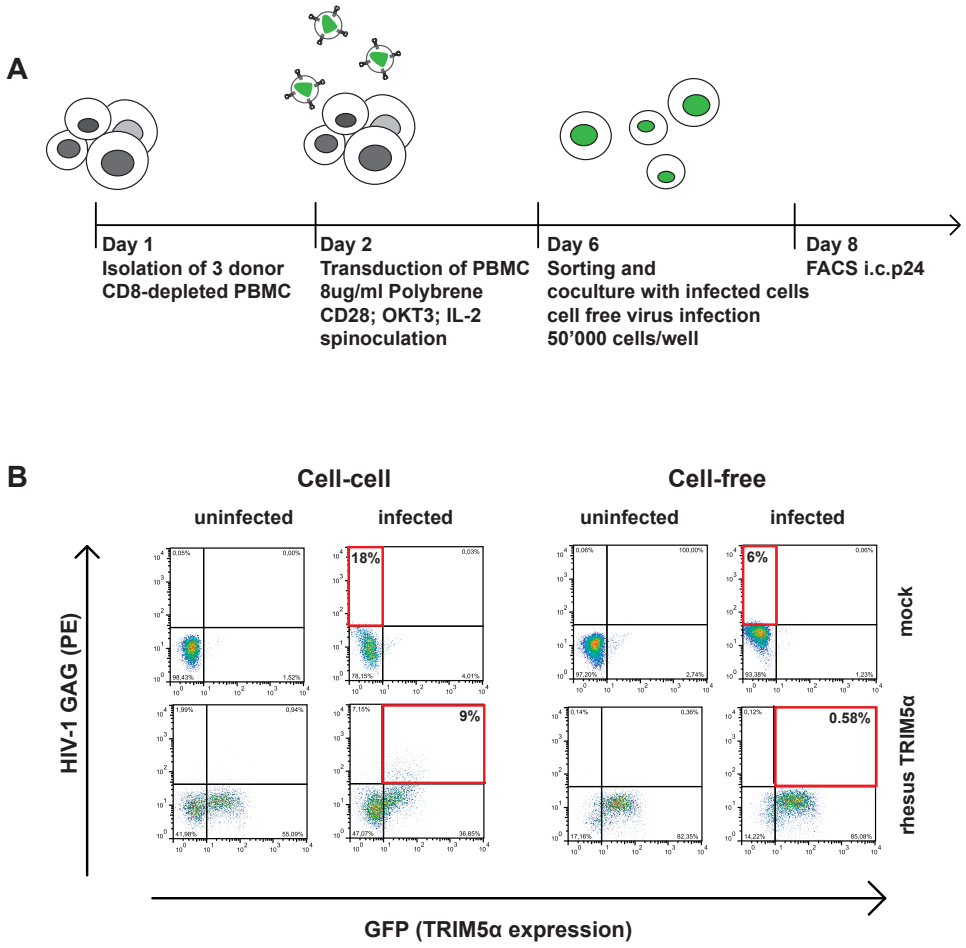

Figure 13. Cell-cell transmission overcomes restriction by rhesusTRIM5α in human PBMC (A) Scheme of the transduction and infection protocol for PBMC. (B) Cell-cell transmission overcomes rhTRIM5α mediated restriction of HIV-1. At day 6 post transduction Uninfected or SF162-infected PBMC (donors) were co-cultivated 1:1 with rhTRIM5a or mock transduced PBMC (mock treated (no gfp), rhTRIM5α (gfp positive)) or challenged with cell-free SF162 at MOI of 0.2. At day 2 post infection (8 days post transduction) infection was assessed by intracellular HIV-1 Gag staining. Data show one representative experiment of two independent experiments.

DISCUSSION

Several enveloped viruses from different families use direct transfer from one cell to another target cell to propagate themselves- and HIV-1 is no exception (Jolly, Kashefi et al. 2004; Groot, Welsch et al. 2008; Sattentau 2008).

The macromolecular structure and organization of the virological synapse has been well investigated and different modes of transmission have been thoroughly characterized (McDonald, Wu et al. 2003; Jolly, Kashefi et al. 2004; Sherer, Lehmann et al. 2007; Groot, Welsch et al. 2008; Sowinski, Jolly et al. 2008; Rudnicka, Feldmann et al. 2009); reviewed in (Sattentau 2008). However, the precise contribution of cell-cell and cell-free virus transmission in the initial infection process and viral spread in vivo remains unclear. Moreover, to what extent neutralizing antibodies are capable of interfering with direct HIV-1 cell-cell transfer, still needs to be unraveled. Considering the postulated importance of cell-cell transmission (Jung, Maier et al. 2002; Pope and Haase 2003; Hladik, Sakchalathorn et al. 2007) the capacity of neutralizing antibodies to act within virological synapses will determine their in vivo efficacy to control viremia.

To analyze the efficacy of neutralizing antibodies in blocking the respective transmission modes, a method to precisely assess cell-cell transmission in vitro is required. So far several attempts have been made to investigate this efficient mode of viral spread either by tracking single fluorescent virions (Chen, Hubner et al. 2007; Hubner, Chen et al. 2007; Hubner, McNerney et al. 2009) or measuring target cell infection (Ganesh, Leung et al. 2004; Massanella, Puigdomenech et al. 2009; Martin, Welsch et al. 2010; Sigal, Kim et al. 2011). However, eliminating one transmission mode without affecting the other still remains a challenge. The efforts made so far to separate these modes of transmission (Sourisseau, Sol-Foulon et al. 2007; Martin, Welsch et al. 2010; Sigal, Kim et al. 2011) are labor intensive and have revealed contradictory results (Ganesh, Leung et al. 2004; Keele, Van Heuverswyn et al. 2006; Chen, Hubner et al. 2007; van Montfort, Nabatov et al. 2007; Massanella, Puigdomenech et al. 2009; Martin, Welsch et al. 2010). One limiting factor in these investigations has been controlling the viral input during cell-cell transmission. Quantifying transferred virus during cell-cell transmission remains difficult as the viral burden of the infected cell cannot be precisely standardized. In contrast, free virus infection is tightly controllable, as it allows the same titered viral stocks to be used in independent experiments. A further drawback of the previously published studies has been the lack of direct comparison of both transmission modes. This has hindered the evaluation of neutralization studies in particular, and resulted in provisional conclusions (reviewed (Sattentau 2010)).

Utilizing the DEAE dependence to assess cell-cell transmission has permitted some control of those factors. Firstly, we were able to successfully reduce cell-free infection to negligible levels by making use of the fact that many CCR5 (R5) using HIV strains are only capable of efficiently infecting engineered, CCR5 and CD4 expressing target cell lines such as TZM-bl in the presence

of polycations (Platt, Wehrly et al. 1998; Wei, Decker et al. 2002; Montefiori 2005). Thus, cell-free HIV-1 infection by JR-FL (Fig.1C) and other R5 using viruses (Fig.2) was dramatically reduced by the omission of DEAE Dextran and the dissection of cell-cell transmission and cell-free infection was feasible. Secondly, through tightly controlled the same virus stocks to measure cell-free infection and to infect donor cells at a low MOI, the input could be precisely titrated for every assay and assured a standardized input in every experiment. This subsequently allowed for a side by side comparison of neutralization efficiency during both transmission modes (Chapter 4.2).

The second approach, which utilizes rhTRIM5α restriction also permitted distinction between both transmission modes. RhTRIM5α is known to efficiently inhibit HIV-1 infection by accelerating capsid disassembly (Perron, Stremlau et al. 2004; Stremlau, Owens et al. 2004) and proteasome independent capsid degradation (Chatterji, Bobardt et al. 2006). While rhTRIM5α efficiently blocks infection by cell-free HIV-1 in A3.01-CCR5, TZM-bl and PBMC (Fig. 9A,10C, 11A and 13) it is considerably less effective at inhibiting cell-cell transmission (Fig.9A, 10D, 11B and 13), which is in line with previous results (Richardson, Carroll et al. 2008). In contrast to the restriction by DEAE omission, which is limited to charge interaction on the cell surface, rhTRIM5α interferes intracellularly with HIV-1 and inhibits cell-free virus infection during another phase of viral entry. How precisely cell-cell transmission overcomes this restriction and whether it is solely a consequence of saturating rhTRIM5α due to high virion concentration at the virological synapse, still needs to be unraveled.

So far, the precise mechanism by which HIV-1 enters the target cells after cell-cell transmission has remained unclear: either fusion directly at the plasma membrane or fusion from within an endosomal compartment, or both (Chen, Hubner et al. 2007; Bosch, Grigorov et al. 2008; Ruggiero, Bona et al. 2008; Hubner, McNerney et al. 2009; Martin and Sattentau 2009). Importantly, our cell-cell assays do not assess virus particle transfer but successful target cell infection independent of the entry pathway. Thus, neutralization results attained with these different assays (Overview table 1) ensure an assessment independent of the distinct HIV-1 entry pathways and restrain cell-free infection at separate levels of the viral life cycle.

In summary, we have developed two experimental systems, which enable direct quantification of cell-cell transmission. Exploiting the DEAE dependence and rhTRIM5α restriction of cell-free infection has allowed exclusive reduction of mode of transmission without affecting the other. The two assays facilitate detection and monitoring of cell-cell transmission using either a standard luminometer or flow cytometry. Moreover, these assays have been used to screen the neutralizing potential of antibodies during cell-cell transmission (Chapter 4.2) and will facilitate future studies of cell-cell transmission neutralization.

Distinction betweeen cell-cell and cell-free transmission modes	Donor cells	Target cells	Virus	Assay readout
DEAE restriction	infected PBMC	TZM-bl	Replication competent virus	Luciferase readout
	transfected 293-T	TZM-bl	pseudotyped virus	Luciferase readout
rhesus TRIM5α restriction	infected PBMC	rh TRIM5α transduced TZM-bl	Replication competent virus	Luciferase readout
	transfected 293-T	rh TRIM5α transduced TZM-bl	pseudotyped virus	
	infected PBMC	rh TRIM5α transduced PBMC	Replication competent virus	i.c. p24 FACS
	infected A3.01-CCR5	rh TRIM5α transduced A3.01-CCR5	Replication competent virus	

Table 1. Overview of developed assay systems. This table summarizes the newly established cell-cell assays. Abbreviations: PBMC, Peripheral blood mononuclear cells; rh, rhesus; i.c. , intracellular staining; FACS, Flow cytometry.

MATERIALS AND METHODS

Reagents

Properties and sources of antibodies and inhibitors used in this study are listed in Table S1. DARPin 57.2 was produced as described (Schweizer, Rusert et al. 2008). T-20 (Wild, Greenwell et al. 1993) was purchased from Roche Pharmaceuticals. Maraviroc (Dorr, Westby et al. 2005) was purchased from Pfizer.

Cells

293-T and HeLa cells were obtained from the American Type Culture Collection (ATCC). TZM-bl cells (Wei, Decker et al. 2002), A3.01 and A2.01 T cells (Coiras, Lopez-Huertas et al. 2010) were obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). All adherenT-cell lines were cultivated in DMEM containing 10% heat inactivated FCS and antibiotics.
A3.01 cells endogenously express CD4 and CXCR4. The sister cell line A2.01 is CD4 negative. CCR5 expressing A3.01 cells (A3.01-CCR5) were generated using retroviral transduction as described ((Platt, Wehrly et al. 1998), C. Gordon, A. Trkola and J.P Moore unpublished data). Suspension cells were cultivated in RPMI containing 10% FCS and antibiotics.
Stimulated peripheral blood mononuclear cells (PBMC) from healthy blood donors were prepared as described (Rusert, Mann et al. 2009) and cultivated in RPMI containing 10%FCS, 100 units per ml IL-2 and antibiotics.

Virus preparation and concentration

Env encoding plasmids of subtype B Tier 1 isolates NL4-3 (X4) (Pandhare and Dash 2011), SF162 (R5) (Cheng-Mayer, Weiss et al. 1989) and Tier 2 JR-FL (R5) (Koyanagi, Miles et al. 1987) were obtained from the NIH ARRRP.
Env–pseudotyped viruses were prepared by co-transfection of 293-T cells with plasmids encoding the respective Env gene and the luciferase reporter HIV vector pNL luc-AM (Gilbert, McKeague et al. 2003) as described (Rusert, Mann et al. 2009). Env pseudotyped particles (pp) generated with this vector are denoted $Env^{pp\text{-}lucAM}$ (e.g. $JR\text{-}FL^{pp\text{-}lucAM}$). Where indicated the corresponding pNL gfp-AM pseudotyping vector (generated by P. Rusert and P. Ocampo) which encodes GFP instead of luciferase was used.
Replication competent (rc) virus subtype B Tier-1 isolates ADA (R5), SF162 (R5), NL4-3 (X4), BZ167 (R5X4), Tier-2 isolates JR-FL (R5), JR-CSF (R5), ZA015, ZA016 and Tier-3 isolate ZA110 (R5) (Rusert, Krarup et al. 2011) were propagated on CD8-depleted PBMC and titered as described (Rusert, Kuster et al. 2005). In experiments where replication competent and pseudotyped virus preparations are compared, viruses are denoted with rc and pp, respectively (e.g. JR-FLrc, JR-FLpp).
All virus preparations were filtered upon harvesting and infectivity and/or p24 content determined to quantify input as described (Rusert, Kuster et al. 2005). For the virus attachment and β-lactamase entry assays virus preparations were concentrated by ultracentrifugation (2 h at 4 °C at 28’000 rpm; swing out rotor SW28, 32% sucrose cushion).

4.2. Cell-cell transmission enables HIV-1 to partially evade inhibition by potent CD4bs directed antibodies (in press)

please note: As this manuscript is finalized and in press the corresponding references are directly at the end of this chapter starting page 85

Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies

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Abstract

HIV is known to spread efficiently both in a cell-free state and from cell to cell, however the relative importance of the cell-cell transmission mode in natural infection has not yet been resolved. Likewise to what extent cell-cell transmission is vulnerable to inhibition by neutralizing antibodies and entry inhibitors remains to be determined. Here we report on neutralizing antibody activity during cell-cell transmission using specifically tailored experimental strategies which enable unambiguous discrimination between the two transmission routes. We demonstrate that the activity of neutralizing monoclonal antibodies (mAbs) and entry inhibitors during cell-cell transmission varies depending on their mode of action. While gp41 directed agents remain active, CD4 binding site (CD4bs) directed inhibitors, including the potent neutralizing mAb VRC01, dramatically lose potency during cell-cell transmission. This implies that CD4bs mAbs act preferentially through blocking free virus transmission, while still allowing HIV to spread through cell-cell contacts. Thus providing a plausible explanation for how HIV maintains infectivity and rapidly escapes potent and broadly active CD4bs directed antibody responses in vivo.

Author summary

HIV is known to spread both in a cell-free state and from cell to cell, however the relative importance of the cell-cell transmission mode in natural infection has not yet been resolved. Design of vaccines attempt to inhibit HIV entry into target cells as do engineered entry inhibitors used as therapeutics. While these agents are known to block the entry of cell-free HIV particles into cells, to what extent cell-cell transmission is vulnerable to such inhibition is unclear. Here we report that the activity of neutralizing antibodies and inhibitors during cell-cell transmission varies depending on their mode of action. A prominent class of neutralizing antibodies directed to the CD4 binding site on the virus envelope very efficiently blocks binding of the virus to its primary receptor on target cells, the CD4 molecule. These types of antibodies are elicited in natural infection and once isolated from infected individuals have shown to be highly potent. Why HIV still replicates in the presence of such potent antibodies remains unclear. Here we show that these CD4 binding site antibodies are dramatically less potent inhibitors of cell-cell transmission, and therefore act preferentially by blocking free virus transmission while allowing HIV to spread through cell-cell contact.

Introduction

The Human Immunodeficiency Virus (HIV) spreads in vitro very efficiently, if not preferentially, by cell-cell contacts. Viral transmission from infected to non-infected cells occurs via formation of virological synapses – organized contact areas which concentrate cellular entry receptors and virions [1-5] - and via transient cell-cell contacts and longer-range intercellular interactions including nanotubes and filopodia [6-8]. Virus transmission through these points of contiguity has been proven in vitro to be more efficient and rapid than infection by cell-free viruses [9-14], supporting the notion that cell-cell transmission may be a relevant if not dominant mode of virus dissemination in infected individuals. The highly efficient transmission of HIV between cells may also foster infection of target cells with multiple virions and so facilitate recombination and escape adaptations to occur more frequently [15-18]. So far the relative contribution of cell-cell and cell-free virus transmission in acquisition of HIV infection and viral dissemination during human infection remain however undefined. This gap in knowledge poses a conceptual problem for neutralizing antibody based HIV vaccine and entry inhibitor design, as it remains uncertain whether both cell-free and cell-cell spread of HIV must be blocked with equal efficacy, or whether only the dominant transmission mode needs to be targeted and if so which.

Neutralizing antibodies recognize epitopes on the envelope glycoproteins gp120 and gp41 that are accessible in the oligomeric form of the HIV envelope protein [19, 20]. Neutralization occurs by blocking virion attachment to host cell receptors or by inhibiting membrane fusion [19]. To date it remains unclear to what extent the relatively enclosed environment of the viral synapse is able to protect the virus from humoral immunity [21, 22]. Previous attempts to determine the capacity of individual neutralizing antibodies to inhibit cell-cell transmission came to varying and conflicting conclusions, suggesting it was entirely inefficient, less efficient than inhibition of cell-free infection, or indeed equally efficient than inhibition of cell-free infection [13, 22-26]. These discrepancies in reported neutralizing antibody efficacy in blocking HIV cell-cell transmission underline the complexity of studying HIV transmission modes and were suggested to likely reflect incongruities amongst cell types studied as well as differences in experimental procedures [21]. A number of studies have shed light on the complexity of HIV transmission modes and revealed substantial differences amongst experimental set ups used to study cell-cell transmission [5, 13, 21, 22, 27]. Cell-associated HIV can be transmitted to uninfected target cells by a variety of modes and may involve both, cells that are productively infected (cis-infection) and cells that trapped virus but remained uninfected (trans-infection [28-30]. Depending on the cell type of the counter partners, their relative frequencies and rate of infection, transmission events can differ on a molecular level and were described to depend on a range of extracellular interaction structures (T-T cell viral synapse [4], DC-T cell viral synapse [3], Macrophage-T cell [31], polysynapses [7], nanotubes [8], filopodia [32] reviewed in [1]). Considering this broad range of potential interactions, it is evident

that monitoring cell-cell transmission, precise quantification of the events, and assessment of inhibitor efficacy has remained most complex. In part conflicting results obtained on neutralizing antibody efficacy in blocking HIV cell-cell transmission [13, 22-26] may be a consequence of the variable types of cell-cell interactions engaged in contacts between cells of different origin as well as differential assay set ups and readouts.

The primary intent of our current study was to derive a definite conclusion on the capacity of neutralizing antibodies to block cell-cell transmission of HIV. Our current knowledge of the mechanism by which antibodies neutralize HIV is largely based on data derived in assay formats which assess cell-free virus infection of a variety of target cells, either in single round or multiple round infection assays [33-38]. While the former assays only monitor free virus entry, the latter measure a composite of free virus and cell-cell transmission during consecutive rounds of replication.
Several types of experimental approaches have been employed to dissect cell-free from cell-cell transmission when infected cells are used as source of virus inoculum. Single virus tracking by confocal microscopy [39, 40], disturbance of cell-cell contacts by keeping cultures in motion [14] and careful time course analysis of virus transmission to restrict analysis to a time window when mostly cell-cell transmission occurs [18, 22, 26]. The latter approach has been the most promising to date. Yet these assays require careful fine tuning of a relatively short infection interval. Virus transfer due to the short interaction can be relatively low, require sensitive detection systems and can be error prone [22]. Here we made use of assay systems which allow overcoming several limitations and explicitly monitoring cell-cell transmission. The comprehensive in vitro analysis of inhibitor activity during cell-free and cell-cell virus transmission that we present here provides a necessary first step towards the definition of the in vivo relevance of the respective transmission modes and ensuing requirements for their inhibition by vaccine induced antibody responses and entry inhibitors.

RESULTS

Quantitative dissection of cell-cell and cell-free transmission of HIV-1

An inherent difficulty in dissecting neutralizing antibody action on cell-free and cell-associated virus is related with the respective assay systems used to evaluate the cell-cell transmission events. While cell-free infection can easily and most precisely be quantified (eg by using single-round infecting viruses), genuine cell-cell transmission is difficult to assess when transmission from infected to uninfected cells is studied. Replication competent virus is required in these settings. Although close cell contacts favor synapse formation and cell-cell transmission [14], entirely excluding the contribution of free virus transmission has thus far remained difficult.

To construct a robust high-throughput system allowing direct comparisons between cell-free and cell-cell transmission we chose the widely used luciferase reporter cell line TZM-bl as target cells [34]. PBMC infected with primary, replication competent (rc) virus isolates served as donor cells in our cell-cell transmission system as these should most closely resemble in vivo infected cells. Direct co-culturing of infected PBMC ($PBMC^{HIV+}$) with TZM-bl cells results in rapid and efficient infection of these cells and can be monitored by induction of the reporter luciferase (Fig.1A).

In order to adapt the $PBMC^{HIV+}$/TZM-bl infection system to specifically quantify cell-cell transmission we made use of the fact that many CCR5 (R5) using HIV strains are only capable of efficiently infecting engineered, CCR5 and CD4 expressing target cell lines such as TZM-bl in the presence of polycations [34, 41, 42]. We found that whilst cell-free infection by the R5 isolate JR-FL was dramatically reduced by the omission of DEAE Dextran (Fig.1B), cell-cell transmission between JR-FL infected PBMC and the TZM-bl target cells was polycation independent (Fig.1A). Free virus released from infected cells in the cell-cell transmission set up failed to infect in absence of polycation (Fig.S1A).

In line with previous reports [9, 11-14, 22], HIV infection kinetics in the $PBMC^{HIV+}$/TZM-bl transmission assay were accelerated compared to free virus infection (Fig.S1B). Of note, regardless of whether cell-free virus adsorption was enforced by spinoculation [43] or magnetic beads [44], entry of cell-free $HIV\text{-}1_{JR\text{-}FL}$ into TZM-bl cells remained severely restricted when no polycation was added (Fig. 1C), reinforcing the notion that enhanced virus transmission during cell-cell contact involves activities that extend beyond a mere increase in membrane proximity. In order to discriminate cell-cell from cell-free virus transmission in the $PBMC^{HIV+}$/TZM-bl infection assay, polycation dependent virus isolates were used (Fig. S2). Input of infected donor cells and cell-free virus input was calibrated so that infection of both occurs in the linear range of the assay system (Fig. 1A and 1B) and that free virus cannot infect in absence of DEAE-Dextran (Fig. 1B and S2A). In sum, these assay conditions allowed precise quantification of cell-cell transmission without interference of free virus infection in the $PBMC^{HIV+}$/TZM-bl infection system.

Mode of virus transmission differentially steers susceptibility to entry inhibitors

Using defined DEAE-Dextran dependent virus isolates (Fig. S2), we next employed the PBMC^{HIV+}/TZM-bl cell-cell transmission assay to evaluate whether the mode of HIV transfer has an influence on the potency of neutralizing antibodies and entry inhibitors. We compared the inhibitory potency of the gp120-directed tetrameric CD4-IgG2 molecule (PRO 542) and the gp41-directed fusion inhibitor T-20 against the isolate JR-FL in cell-free and cell-cell virus transmission. Strikingly, inhibition of cell-cell transmission by CD4-IgG2 required an approximately 40-fold higher 50% inhibitory dose than inhibition of the same virus strain during cell-free infection (Fig. 1D). In contrast, the gp41 directed fusion inhibitor T-20 was markedly less affected by the transmission mode requiring only 3-fold higher IC50 doses during cell-cell transmission. To verify whether the decreased sensitivity towards CD4-IgG2 during cell-cell transmission was merely due to more efficient adsorption of the virus to the target cells or an inherent feature of cell-cell transmission, we assessed the inhibitory capacity of CD4-IgG2 and T-20 against cell-free virus adsorbed to target cells by spinoculation or by magnetic beads (Fig. 1E). Both inhibitors remained equally active regardless whether adsorption of cell-free virus was increased or not, indicating that indeed cell-cell transmission associated events caused the loss of CD4-IgG2 activity rather than simple virus concentration on the target cell surface.

To restrict our assessment to the first round of cell-cell transmission events we next probed the efficacy of CD4-IgG2 and T-20 against cell-free JR-FL envelope pseudotyped virus (pseudovirus particle, pp) and in cell-cell transmission using 293-T cells transfected with plasmids encoding JR-FL pseudotyped virus as donor cells (Fig. 1F). Like replication competent virus, the JR-FLpp proved more resistant to CD4-IgG2 inhibition during cell-cell transmission and required 190-fold higher concentrations to achieve 50% inhibition than cell-free pseudo-virus (Fig. 1F).

Gp120 specific entry inhibitors have decreased capacity to block cell-cell transmission

Our initial observations of the distinct effect of cell-cell transmission on CD4-IgG2 and T-20 activity raised the question whether the epitope specificity or neutralization mechanism of a given inhibitor determines its activity during cell-cell transmission of HIV. To probe this we investigated a panel of well characterized neutralizing antibodies and entry inhibitors for their respective potencies against cell-free and cell-associated virus. We selected inhibitors based upon their mode of action: cell- directed (CD4 or coreceptor CCR5 blocking; Fig 2A), virus directed (gp120 (Fig 2B) and gp41 specific (Fig 2C)). Whenever possible inhibitors that differ in molecular mass and

chemical structure (peptide, small molecule inhibitor, antibody) were included for comparison (Table S1).

Comparison of the inhibitor activity under the two transmission modes revealed an intriguing pattern (Fig. 2D). While cell-directed inhibitors (anti-CD4, anti-CCR5) blocked cell-cell and cell-free transmission of JR-FL with almost identical efficacy (Fig. 2A and D, <4-fold loss of activity), HIV-1 envelope directed inhibitors showed a remarkably dichotomous pattern (Fig. 2B-D). All CD4 binding site specific agents (mAbs b12, VRC01, 1F7, the tetrameric CD4-IgG2 molecule and the CD4 mimetic CD4M47 [45]) lost considerable potency when cell-cell transmission occurred (10 to 100 fold decrease in activity reflected in according increase in IC50). Of particular note were the results we obtained for mAb VRC01. While VRC01 is one of the most potent antibodies in inhibiting cell-free transmission described to date [46, 47], it proved particularly ineffective in inhibiting cell-cell transmission of JR-FL. Similarly to the CD4bs specific agents, the carbohydrate specific mAb 2G12 also lost considerable activity when blocking of cell-cell transmission was required. This was in sharp contrast to the gp41 specific agents, the MPER-targeting neutralizing antibodies 2F5 and 4E10 and the fusion inhibitor T-20 which were all only marginally affected by the mode of virus transmission (Fig. 2C). Particularly surprising were the activities of the two MPER specific mAbs, despite the fact that they are not potent inhibitors of cell-free JR-FL virus transmission, their ability to block cell-cell transmission remained in the same range.

The data we obtained thus far supported the notion that virus directed entry inhibitors fall into two distinct classes with a differential activity during cell-cell transmission: such that lose potency (eg CD4bs directed agents) and such which appear largely unaffected in their activity irrespective of the virus transmission mode (gp41 directed agents). We next verified the differential activity of specific CD4bs directed agents (CD4-IgG2 and VRC01) and the gp41 directed agents (2F5, 4E10 and T-20) in cell-cell and free virus transmission using four genetically divergent viruses, the Tier-1 virus ADA, the Tier-2 isolates ZA015, ZA016 and the Tier-3 isolate ZA110 (Fig. 3). The same pattern of reactivities was also seen for these viruses: CD4bs directed agents lost substantial potency during cell-cell transmission, while MPER mAbs and T-20 were only marginally affected (<4-fold for MPER mAbs).

Efficient inhibition of T-cell to T-cell transmission by gp41 directed inhibitors

While the PBMC^{HIV+}/TZM-bl assay proved very robust and provides a means to study cell-cell transmission under tightly controlled conditions, it has two major limitations. For one, only R5 viruses which depend on polycation in order to infect the engineered target cells can be studied. Secondly, while TZM-bl cells are widely used as target cells in HIV neutralization assays, they are

of epithelial origin and engineered to express CD4 and CCR5 in abundance [41]. Considering that type and densities of cellular receptors engaged in forming the virological synapse may differ to some extent depending on the types of cells engaged, we thought it prudent to verify our observations in a setting of T-cell to T-cell transmission.

To this end we employed an alternate assay system making use of the intracellular HIV restriction factor TRIM5α. While HIV has adapted to human TRIM5α (huTRIM5α), HIV infection is potently restricted by rhesus macaque TRIM5α (rhTRIM5α) which acts post-entry at steps preceding integration [48-50]. Notably this restriction appears to limit cell-free virus infections, but not cell-cell transmission [51]. We made use of this selective action of rhTRIM5α and generated A3.01-CCR5 T cells which co-expressed GFP and either huTRIM5α or rhTRIM5α as described [51]. While the parental A3.01-CCR5 T cells are permissive for HIV and cells transduced with huTRIM5α remained permissive, rhTRIM5α expression rendered the A3.01-CCR5 T cells highly resistant to infection by cell-free virus (Fig. S3A) but not to infection by HIV via the cell-cell transmission route (Fig. S3B). Most importantly for our transmission studies rhTRIM5α restriction of cell-free infection occurs irrespective of coreceptors usage (Fig. S3A) and hence allows measurement of cell-associated virus transmission with a wider spectrum of virus isolates.

To probe the effect of entry inhibitors in T-cell to T-cell transmission we performed inhibition assays using HIV infected A3.01-CCR5 cells (A3.01-CCR5^{HIV+}) as donor cells and rhTRIM5α expressing A3.01-CCR5 cells as targets (A3.01-CCR5^{rhTRIM5α}) (Fig. 4A and B). We observed the same pattern of virus specific entry inhibition as in the PBMC^{HIV+}/TZM-bl assay (Fig. 2). Gp41 directed inhibitors had similar or only slightly reduced activities in inhibiting the Tier-1 viruses SF162 (R5) and NL4-3 (X4) and the Tier-2 isolate JR-CSF (R5) during cell-cell transmission. In contrast CD4bs directed agents lost again considerable potency during cell-cell transmission. The V3 loop specific neutralizing antibody 447-52D [52] showed a strain dependent pattern. While 447-52D inhibition of NL4-3 was decreased from 90% to 14% during cell-cell transmission, only a marginal loss of SF162 inhibition occurred. Cell-free NL4-3 and SF162 is inhibited by 447-52D with similar potency [53], suggesting that differential V3 loop exposure during the entry process steers the efficacy of the mAb during cell-cell transmission, rather than higher potency. In line with this a second V3 loop antibody 1-79 [54] also blocked cell-free and cell-cell transmission of SF162 with identical potency (Fig.S4A).

To verify our findings in a setting where transmission was studied solely on primary T cells, we generated rhTRIM5α expressing PBMC and monitored their infection by cell-free virus and cell-associated virus using HIV infected PBMC (Figure 4C). The data obtained in the PBMC^{HIV+}/PBMC^{rhTRIM5α} assay confirmed our findings in the PBMC^{HIV+}/TZM-bl and A3.01-CCR5^{HIV+}/A3.01-CCR5^{rhTRIM5α} assays, and showed decreased activity of CD4bs antibodies, strain dependent reduction of V3 mAb inhibition and comparable activity of gp41 directed inhibitors during cell-cell transmission.

Capacity to interfere with HIV attachment to target cells is not a prerequisite for neutralizing antibodies to block cell-cell transmission

Our analysis of entry inhibitor activity in cell-cell transmission thus far had revealed a dichotomous pattern for virus envelope directed agents. While most gp120 directed agents, and in particular CD4bs agents, suffered from a considerable loss in activity during cell-cell transmission, gp41 directed inhibitors maintained their activity. We hypothesized that the basis for this dichotomy could be a genuine difference in inhibition modes and that the capacity to inhibit a specific phase of the entry process determines efficacy in blocking cell-cell transmission. To explore this we first evaluated the capacity of neutralizing antibodies to block attachment of fluorescently labeled HIV to target cells during spinoculation (Fig. 5A). Within this setup virus binding to a variety of cell lines and PBMC proved to be predominantly driven by binding of virions to CD4 (Fig.5B). In contrast to previous reports [55], only marginal attachment of HIV to CD4 negative cells was detected in our assay set up. CD4 independent attachment of HIV to target cells was previously found predominantly amongst X4 isolates which were shown to bind cell surface expressed glycosaminoglycans (GAG) when target cells were incubated with concentrated virus stocks at 37°C [55, 56]. Our current analysis required assessment of attachment in a setting where binding of virions to cells is both, synchronized and entry halted before fusion. We achieved this by using spinoculation and a temperature arrest at 23°C and found that under these conditions non-CD4 driven attachment is negligible.

In line with the CD4 dependence in the attachment assay, CD4bs directed inhibitors, b12, VRC01 and CD4-IgG2 potently inhibited binding of JR-FL (R5) and NL4-3 (X4) to target cells (Fig.5C and 5D). Interestingly, the V3 loop specific mAb 447-52D possesses a partial activity in inhibiting attachment of NL4-3 to target cells, suggesting that in some virus/antibody pairings co-receptor engagement may play a role in establishing firm attachment. In contrast MPER-directed antibodies 2F5 and 4E10 and the fusion inhibitor T-20 were not able to inhibit attachment. The latter is in accordance with the previously described limited capacity of MPER mAbs to neutralize virions before CD4 engagement [38, 57, 58].

Neutralizing antibodies with post-attachment activity maintain potency during cell-cell transmission

Following gp120 binding to CD4, HIV-1 enters its target cell in a multistep, temporally defined process (reviewed in [59]). In order to measure the inhibitory capacity of neutralizing antibodies on virus entry at two different stages of the infection process, we assessed virus-cell fusion utilizing β-lactamase (BlaM) loaded virions as described [60, 61] (Fig. 6A). Inhibitors were either

added before HIV attachment to target cells and were removed following spinoculation or alternatively added after HIV attachment to target cells, hence providing a method by which post-CD4 engagement inhibitory activity can be measured. As expected when inhibitors were added before virion binding to PBMC or A3.01-CCR5 target cells and were present throughout the attachment process, all probed compounds potently blocked virus fusion (Fig. 6B and C). However, addition of inhibitors after attachment of the virus to the target cells, revealed a dichotomous pattern. CD4bs reactive agents completely lost their activity while the MPER-specific antibodies and the gp41-directed fusion inhibitor T-20 still possessed substantial inhibitory activity. The capacity of the gp41-directed agents to block infection post CD4 recruitment, is in line with previous reports observing MPER mAbs and HR1 and HR2 targeting inhibitors which act at a prefusion stage [57, 58, 62-66].

This divergent pattern of reactivities of gp120 CD4bs and gp41 directed agents in the fusion assay paralleled their capacity for inhibition of cell-cell transmission, raising the possibility that neutralizing activity post-CD4 engagement is required for efficient blocking of cell-cell transmission.

To resolve pre- and post-attachment activity of inhibitors in more detail, we infected A30.1-CCR5 cells with envelope pseudotyped luciferase reporter viruses again performing treatment with neutralizing antibodies before or after attachment of the virus particles to the cells (Fig. 7A). For all four pseudo-viruses probed (NL4-3, SF162, JR-FL and 6535) the potency of gp120 CD4bs directed reagents (CD4-IgG2, CD4M47 and mAb b12) was dramatically reduced when added after receptor engagement (Fig. 7B). Since the peptidic inhibitor CD4M47 experienced the same difficulties in blocking cell-cell transmission as the CD4bs antibody and CD4-IgG2 despite its small size, the limited capacity of mAbs to access the CD4bs during cell-cell transmission is unlikely to be responsible for their reduced activity during cell-cell transmission. In contrast to CD4bs agents, the potency of the gp41-directed inhibitor T-20 and the MPER-specific antibodies remained essentially unchanged when added after receptor engagement. The V3 loop mAb 447-52D showed an intermediate pattern, it lost more than 50% of its activity against NL4-3 and SF162, but activity against the isolate 6535 was preserved, indicating again that V3 loop exposure post CD4 binding varies in a strain dependent manner. Importantly, strain 6535 was inhibited with identical activity by 447-52D during cell-cell transmission (Fig.S4B). The same was true for the V3 loop mAb 1-79 which blocked SF162 potently post attachment (Fig.7C) and during cell-cell transmission (Fig. S4A).

Anti-CD4 directed agents (CD4-DARPin 57.2, anti-CD4 mAbs OKT4A [67] and 13B8.2 [68]) showed decreased activity when added post attachment, while anti-CCR5 inhibitors (AD101, PRO140, PSC-RANTES) had, in most cases, comparable activity when present before and after attachment (Fig. 7C). This observation suggests that under these assay conditions coreceptor engagement has not been fully established prior to inhibitor addition. The decreased activity of the anti-CD4 inhibitors in the post-attachment assay is expected from their mode of action. These

CD4 receptor directed agents nevertheless block free-virus transmission and cell-cell transmission equally well, while CD4bs gp120 directed inhibitors do not. This highlights the advantage cell-directed inhibitors have, as their target is accessible before and during envelope attachment. In contrast virus-directed inhibitors only have a narrow window of opportunity to act - after virus envelope proteins are expressed and transported to the surface of infected cells.

DISCUSSION

The primary aim of our current study was to dissect the efficacy of neutralizing antibodies and entry inhibitors in the context of cell-cell transmission of HIV. It is generally agreed that neutralizing antibody responses will be a key component of an effective HIV vaccine [69, 70]). However, whether vaccine elicited neutralizing antibodies will need to block only the infecting inoculum, or whether protection will also require restriction of consecutive rounds of infection, and hence inhibition of both cell-free and cell-cell transmission with equal efficacy is not known.

Likewise, in established infection, should one of the transmission modes prove to be clearly dominant, this mode may need to be targeted preferentially by therapeutic vaccines and entry inhibitors.

While it is known that HIV spreads highly efficiently through various types of cell-cell contacts [9-14], so far no consistent picture of antibody action during this process has emerged [13, 22-26]. Only few neutralizing antibodies and inhibitors have been probed for their efficacy in cell-cell transmission, amongst these are antibodies and inhibitors related to those used in our current study (b12 [71], 447-52D [52], 2F5 [72], 4E10 [73, 74]; anti-CD4 mAbs Leu3a and Q4120 [75], and anti-coreceptor inhibitors AMD3100 [76], TAK779 [77]). Several studies reported that MPER mAbs [13], CD4bs mAbs [13, 24], T-20 [13], and anti-coreceptor agents [4, 13] were significantly less potent inhibitors of cell-cell transmission than cell-free virus transmission. Others found cell-cell and cell-free neutralization activity to be equivalent (MPER mAbs [22, 25, 26], CD4bs mAbs [26], V3 mAb 447-52D [4], fusion inhibitors T-20 [22] and C34 [26], anti-CD4 agents [4, 22, 26, 40], and anti-coreceptor agents [22, 39]). However the wide range of assay systems used adds complexity to the interpretation and comparison of the results. Several experimental approaches did not allow direct comparison of cell-cell and cell-free transmission in the same setting [4, 13, 25, 26, 39], other assay systems do not allow precise quantification of inhibitory activity [4, 23, 25]. Additionally, all systems employed to study genuine cell-cell transmission thus far are technically challenging and can be error prone [22]. Nevertheless, in agreement with our observations, one study also reported lower activity of the CD4bs mAb b12 during cell-cell transmission, albeit this difference was rated as non significant by the authors [22].

Here we report on neutralizing antibody activity during cell-cell transmission using specifically tailored experimental strategies which enable unambiguous discrimination between the two trans-

mission modes. The principle by which free virus infection can be distinguished from cell-cell transmission in these two systems is different. In the TZM-bl transmission assay free virus infection of specific R5 viruses can be restricted by omission of the polycation DEAE-Dextran in the infection medium (Fig1B and S2). In the second assay system we make use of the capacity of the restriction factor rhesus TRIM5α which potently interferes with free virus infection but not cell-cell transmitted virions (Figure S3) [51]. While the mode and stage of free virus restriction in the two assay systems are different, the outcome of our analysis of neutralizing antibody capacity in both systems was identical. Gp120 directed inhibitors and neutralizing antibodies, in particular CD4bs directed agents, showed markedly decreased potency in blocking cell-cell transmission, whereas the probed gp41 directed inhibitors, the fusion inhibitor T-20 and the MPER antibodies 2F5 and 4E10, demonstrated identical or only marginally reduced potency. Of particular note are the cell directed inhibitors. Both CCR5 and CD4 targeting compounds were equally active during both transmission modes (Fig. 2A). Although they target the same step in virus entry, namely gp120 interaction with CD4, we found that anti-CD4 inhibition was not decreased in cell-cell transmission, while CD4bs mAb activity is markedly lower (Fig. 2B and 4BC). This is not unexpected. During cell-cell transmission, inhibitors targeting the cellular receptors have a clear advantage, CD4 and CCR5 receptors are always accessible on target cells and inhibitors can bind immediately. In contrast, virus directed inhibitors depend on the initiation of the viral synapse formation, as only then the viral envelope becomes accessible. As the viral synapse formation is tightly linked to gp120 binding to CD4 [3, 4, 13], CD4bs specific inhibitors are likely to only have a narrow time window for action as evidenced by their loss of activity during cell-cell transmission.

Only a limited number of entry inhibitors have thus far been probed for their activity during chronic infection in animal models, and even fewer have reached clinical investigation in therapeutic settings. At present only two inhibitors, the CCR5 specific inhibitor Maraviroc and the fusion inhibitor T-20 are in clinical use. Intriguingly, the currently available data may point towards a potential link between inhibitor action in established infection in vivo and activity during cell-cell transmission. The capacity of Maraviroc and T-20 to restrict cell-free and cell-cell transmission with equal efficacies, and the comparative failure of CD4-IgG2 to do the same, parallels their differential clinical success [78-83]. It is tempting to speculate that potent inhibition of cell-cell transmission is a prerequisite for the therapeutic success of entry inhibitors during established infection. This would bode well for the development of cell-directed inhibitors as all CD4 and CCR5 directed inhibitors we probed potently blocked cell-cell transmission. Importantly this would also imply that cell-cell transmission is responsible for a substantial proportion of viral spread in infected individuals.

Will activity against free virus suffice for both prophylactic vaccine induced antibody responses and prophylactic interventions with entry inhibitors? Or is inhibition of cell-cell transmission also required in these settings? Judging from the success of animal protection/challenge studies performed with b12 [84, 85], a mAb which we find does not inhibit cell-cell transmission efficiently,

one could speculate that potent activity against cell-cell transmission may not be necessary for prophylactic vaccines. Yet, a dual function of antibody based vaccines against both incoming cell-free virus and early cell-cell transmission could potentially enhance efficacy. Based on our observations we consider it thus prudent to incorporate cell-cell transmission studies in current pre-clinical and clinical vaccine assessment to determine whether or not activity in blocking cell-cell transmission is a correlate of protection.

It is very intriguing that amongst the neutralizing antibodies probed the activity of the MPER mAbs and T-20, were the least affected by the virus transmission modes. 2F5 and 4E10, in comparison, displayed only modest potency in free virus inhibition. Yet this activity was largely maintained during cell-cell transmission suggesting that the window of opportunity of action for these mAbs is similar during both entry processes. Several lines of evidence support this hypothesis. MPER specific antibodies can bind and neutralize free virions before receptor engagement [38, 86], however this process is slow, requiring several hours. In contrast these mAbs appear to act preferentially in a cellular context following HIV envelope engagement by CD4. Once the envelope trimer has bound cellular receptors and envelope rearrangements proceed, the MPER domain becomes more accessible allowing the antibodies to rapidly bind and neutralize the virus [57, 58, 86-89]. In line with this we found that MPER mAbs were not able to inhibit attachment, but blocked fusion, both when added during or after attachment (Figs 5-7). Thus MPER mAbs and T-20 can block virus that has already bound to receptors, highlighting that processes required for the transition from receptor engagement to fusion are slow enough for these agents to act. Most importantly in the context of our current study, this suggests that the timing of these processes is identical, regardless of whether free virus or cell-cell transmitted virus is concerned. Post-CD4 attachment activity was previously also reported for other neutralizing antibodies besides MPER mAbs and gp41 targeting inhibitors including V3 loop mAbs and small molecule inhibitors targeting CCR5 [64-66, 90], supporting our observations.

Inhibitors targeting the cellular receptor have immediate access to CD4 and the coreceptors both during cell-cell and cell-free transmission, corresponding to the identical activity we observed in both settings. In turn this highlights that the binding of gp120 to CD4 in the cell-cell transmission setting must be the rate limiting step for CD4bs directed agents. Virus specific antibodies and inhibitors, regardless of the epitope they recognize and their actual size, can only reach the virus envelope once it becomes exposed on the cell surface. When infected cells are in close proximity to potential target cells it is likely critical for neutralizing antibodies to reach the exposed envelope proteins in time, before they encounter target cell receptors and cell-cell transmission commences.

Once the trimer becomes accessible, CD4 engagement appears to be initiated too rapidly for CD4bs specific agents to block with equal efficacy as in cell-free virus transmission. We found that activity of other gp120 antibodies during cell-cell transmission such as 2G12 or V3 loop spe-

cific mAbs, which arrest virus infection post-CD4 engagement, can also be substantially reduced during cell-cell transmission. Interestingly for V3 loop specific antibodies we noted a differential activity during cell-cell transmission depending on the mAb and virus strain investigated (Figs. 4B, 4C and S4). How quickly a given virus envelope proceeds from CD4 engagement to coreceptor binding and fusion will likely be determined by its intrinsic reactivity [91] which may also influence activity during cell-cell transmission. The more rapid this process the less effective the respective antibody will likely be in blocking cell-transmission. In line with this we found that mAb 447-52D blocks virus strains NL4-3, SF162 and 6535 potently when present during attachment, but only strain 6535 at comparable levels when added post attachment. SF162 was also inhibited with identical potency when added during pre- and post attachment by the V3 loop mAb 1-79. Importantly in all cases we investigated, high post-attachment activity of V3 loop mAbs was associated with high efficacy in inhibiting cell-cell transmission (Figs. 4B, 4C, S4 and 7).

A key finding of our study is the failure of CD4bs specific antibodies to maintain their potency during cell-cell transmission. A wealth of data on this antibody category has emerged over recent years. CD4bs specific antibodies are ubiquitously elicited during natural infection [46, 92-95], subject to escape [96], and undergo substantial somatic hypermutation to adapt [54, 94, 97], which can lead to the generation of broadly active, potent neutralizing CD4bs specific antibodies [46, 71, 94]. There is recent evidence that the evolution of potent neutralizing antibodies may follow similar paths across individuals and from different immunoglobulin heavy genes [94].
HIV escapes antibody responses rapidly [98-102]. Accordingly, even the most potent and broadly active antibodies characterized in recent years [46, 47, 94, 103-106] have nonetheless been isolated from individuals who fail to control viremia. However due to their breadth and potency these mAbs may indeed prove to be the responses required for an effective, prophylactic vaccine. Nevertheless their failure to halt disease progression needs to be understood. Our observations may resolve the conundrum of how CD4bs mAbs can be so exorbitantly powerful in vitro and yet to our current knowledge lack comparable potency in vivo and fail to suppress viremia to undetectable levels for prolonged periods. We show here that the blocking activity of CD4bs antibodies is largely directed towards free virus, thereby restricting virus spread to the cell-cell route. In the resultant setting their blocking activity is vastly reduced, thus allowing virus replication and spread to occur. Simultaneously this partial inhibition scenario likely fosters escape as sufficient replication under a partial selection pressure is maintained. In vitro and in silico studies of drug resistance evolution which factored in cell-cell transmission recently came to similar conclusions [18]. The continuous selection of virus escape variants, the high somatic hypermutation of CD4bs antibodies and the emergence of highly potent CD4bs directed neutralizing antibodies underline that these antibodies are continuously imposing a selection pressure on the virus.
In support of our findings, Poignard and colleagues previously observed that high serum concen-

trations of b12 and other neutralizing monoclonal antibodies, which provide protection against free virus challenge, lose their impact in an ongoing established infection in hu-PBL-SCID mice [107]. Of particular interest, b12 resistant virus rapidly emerged while wildtype, neutralization sensitive virus was maintained concurrently, a finding which corresponds to our proposed scenario where HIV may in part escape neutralization and maintain infection by cell-cell transmission.

Based on our observations it is tempting to speculate on the in vivo relevance of cell-free and cell-cell transmission. We hypothesize that the selection pressure provided by CD4bs mAbs should be stronger on free virus than on cell-cell transmitted virus. The fact that CD4bs antibodies can nevertheless maintain an apparently considerable and continuous selection pressure in vivo would argue in turn that free virus transmission must be an important component of viral spread in infected individuals. The importance of free virus transmission may thereby lie either in a quantitatively higher contribution to viral spread in the infected individual or a qualitative asset. Should virus spread preferentially occur through neutralizing antibody vulnerable free virus transmission, cell-cell transmission would allow the virus to maintain replication despite antibody pressure and foster rapid escape. Alternatively, should cell-cell transmission constitute a higher proportion of transmission events in vivo, we would argue that free virus transmission must nevertheless be important, otherwise the selection pressure on free virus transmission could not be so pronounced. It is likely that in the latter case cell-cell transmitted viruses still depend on free virus transmission to reach anatomically distant sites. This may also be crucial for dissemination of the virus in as target cell availability at the initial sites of replication will decrease. However, should cell-cell transmission indeed be the quantitatively dominant transmission mode, it is feasible that antibody responses which specifically restrict this transmission mode could emerge. With the panel of antibodies probed in our current study we saw preferential blocking of free virus not cell-cell transmission. It will be intriguing to probe larger antibody panels in future studies and to determine to what extent the recently defined, potent quaternary and carbohydrate specific mAbs [103, 104] inhibit cell-cell transmission. Common selection processes probe free virus transmission thus may not have detected antibodies targeting cell-cell transmission. Defining whether antibodies that preferentially target the cell-cell transmission exist, should aid resolution of the relative importance of this transmission mode and its inhibition.

Regardless of which scenario holds true, we would argue that cell-cell transmission and the ensuing virus production from infected cells cannot be scarce otherwise viremia levels would drop more dramatically during those periods when the autologous CD4bs specific neutralization response is effective and restricting free virus transmission. Of note, viral set points in chronic infection, while comparatively stable, nevertheless fluctuate, commonly within a 0.5 to 1 log range [108]. It is tempting to hypothesize that this fluctuation may be in part the result of alternating periods of effective neutralization of free virus by the autologous neutralization response, during which only cell-cell transmission occurs, followed by periods where the virus has escaped the

neutralization response and both transmission modes are effective.

In sum our analyses provide compelling evidence that neutralizing antibodies, depending on their mode of action, differ in their capacity to block free virus and cell-cell transmission. According to current knowledge HIV relies on both transmission modes to maintain infection in vivo. We therefore argue that the efficacy of entry inhibitors and neutralizing antibodies to block cell-cell transmission needs to be considered.

Abbreviations

BlaM, β-lactamase

DEAE, diethylaminoethyl

IC50, 50% inhibitory concnetration

pp, pseudo particle

rc, replication competent

RLU, relative light unit

MATERIALS AND METHODS

Ethics statement

PBMC were purified from buffy coats from anonymous blood donations from healthy individuals obtained by the Zurich Blood Transfusion Service (http://www.zhbsd.ch/) under a protocol approved by the local ethics committee.

Reagents

Properties and sources of antibodies and inhibitors used in this study are listed in Table S1. DARPin 57.2 was produced as described [109]. T-20 [110] was purchased from Roche Pharmaceuticals. Maraviroc [111] was purchased from Pfizer. CD4M47 was synthesized as described [45] and kindly provided by J. Robinson.

Cells

293-T and HeLa cells were obtained from the American Type Culture Collection (ATCC). TZM-bl cells [34], A3.01 and A2.01 T cells [112] were obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). All adherent cell lines were cultivated in DMEM containing 10% heat inactivated FCS and antibiotics.

A3.01 cells endogenously express CD4 and CXCR4. The sister cell line A2.01 is CD4 negative. CCR5 expressing A3.01 cells (A3.01-CCR5) were generated using retroviral transduction as described ([41], C. Gordon, A. Trkola and J.P Moore unpublished data). Suspension cells were cultivated in RPMI containing 10% FCS and antibiotics.

Stimulated peripheral blood mononuclear cells (PBMC) from healthy blood donors were prepared as described [36] and cultivated in RPMI containing 10%FCS, 100 units per ml IL-2 and antibiotics.

Virus preparation and concentration

Env encoding plasmids of subtype B Tier 1 isolates NL4-3 (X4) [113], SF162 (R5) [114] and 6535 (R5) [115] and Tier 2 JR-FL (R5) [116] were obtained from the NIH ARRRP.

Env–pseudotyped viruses were prepared by co-transfection of 293-T cells with plasmids encoding the respective Env gene and the luciferase reporter HIV vector pNLluc-AM [117] as described [36].

Env-pseudotyped particles (pp) generated with this vector are denoted $Env^{pp\text{-}lucAM}$ (e.g. JR-FLpp-lucAM). Where indicated the corresponding pNLgfp-AM pseudotyping vector (generated by P. Rusert and P. Ocampo) which encodes GFP instead of luciferase was used. Env- pseudotyped particles generated with this vector are denoted $Env^{pp\text{-}gfpAM}$ (e.g. JR-$FL^{pp\text{-}gfpAM}$).

Replication competent (rc) virus subtype B Tier-1 isolates ADA (R5), SF162 (R5), NL4-3 (X4), BZ167 (R5X4), Tier-2 isolates JR-FL (R5), JR-CSF (R5), ZA015, ZA016 and Tier-3 isolate ZA110

(R5) [53] were propagated on CD8-depleted PBMC and titered as described [118]. In experiments where replication competent and pseudotyped virus preparations are compared, viruses are denoted with rc and pp, respectively (e.g. JR-FLrc, JR-FLpp).

Replication competent virions were GFP labeled by two alternate procedures. We used the full length replication competent NL4-3 derived HIV-GagiGFP vector [13]. Alternatively, virions were labeled by incorporation of chimeric vpr-GFP as described [119]. To this end 293-T cells were co-transfected with a plasmid encoding a full length molecular clone of HIV (TN8 NL [120] and the plasmid pEGFP-Vpr (gift from B. Paxton).

Alternatively, to obtain GFP labeled pseudoparticles, an Env gene deleted pseudotyping vector was generated from the full length replication competent HIV-GagiGFP vector [13]. Briefly, envelope from the HIV-GagiGFP construct was replaced by the corresponding env-deleted luciferase expressing sequence from pNLluc-AM via XhoI and EcoRI. This vector (HIV-iGFP) was then used to generate Env-pseudotyped particles by cotransfecting 293-T cells together with the desired envelope encoding plasmid. Env-pseudotyped particles generated with this vector are denoted Env^{ppiGFP} (e.g. JR-FLppiGFP).

Replication competent β-lactamase labeled viral particles NL4-3rc-BlaM, were generated by co-transfecting the pCMV4-3BlaM-Vpr plasmid (gift from W. C. Greene), plasmid pAdVAntage (Promega) and the replication competent proviral vector TN8 as described [61]. To generate BlaM-vpr labeled JR-FL env pseudoviruses (JR-FLpp-BlaM) 293-T cells were co-transfected with plasmids pCMV4-3BlaM-Vpr, JR-FL env and pNLluc-AM.

All virus preparations were filtered upon harvesting and infectivity and/or p24 content determined to quantify input as described [118]. For the virus attachment and β-lactamase entry assays virus preparations were concentrated by ultracentrifugation (2h at 4°C at 28'000 rpm; swing out rotor SW28, 32% sucrose cushion).

Generation of TRIM5α expressing cells

Bicistronic lentiviral GFP and TRIM5α expression vectors huTRIM5α or rhTRIM5α [51] were provided by J.L.Riley. Lentiviral vectors were produced upon co-transfection of 293-T cells with the TRIM5α encoding vector, the VSV envelope encoding plasmid pHEF-VSVG [121] obtained through the NIH ARRRP) and the packaging plasmid pCMV-dR8.91 ([122]; gift from D. Trono). A3.01-CCR5 cells were transduced by spinoculating (2h at 1200g) 100 lentiviral particles per cells in DMEM containing 10% FCS, antibiotics, and 10μg/ml DEAE. PBMC were transduced one day after isolation and stimulation by spinoculation (2h 1200g) with 800 lentiviral particles in RPMI containing 10% FCS, antibiotics and 8μg/ml Polybrene. PBMC Transduced PBMC were cultured on 48 wells coated with OKT3 and 2μg/ml CD28 and TRIM5α positive cells were retrieved by

FACS sorting on day 4 after transduction. Expression of huTRIM5α or rhTRIM5α was monitored by detection of bicistronic expressed GFP by FACS.

Assessment of free virus and cell-cell transmission in the PBMC^{HIV+}/TZM-bl infection system

We developed an assay system based on infection of TZM-bl cells, which allows easy and quantitative discrimination between cell-free and cell-cell transmission. This is possible as many R5 viruses depend on polycationic supplements in the cell culture medium in order to infect TZM-bl cells as cell-free virions but not during cell-cell transmission (Figure 1).

For cell-free virus infection, the neutralization activity of mAbs and inhibitors was evaluated on TZM-bl cells essentially as described using replication competent virus as inoculum [36]. Cell-free, replication competent virus input was chosen to yield virus infectivity corresponding to 5'000-10'000 relative light units (RLU) per 96 well in absence of inhibitors. Cell-free virus infections were carried out in culture medium containing 10µg/ml of the polycation DEAE (diethylaminoethyl; Amersham Biosciences, Fairfield, Connecticut, USA) if not otherwise indicated.

To assess cell-cell transmission and inhibition thereof, stimulated CD8-depleted peripheral blood mononuclear cells (PBMC) from healthy blood donors were infected with replication competent virus stocks at a MOI 0.01. Cell-cell transmission in the PBMC^{HIV+}/TZM-bl infection system had the same linear dynamic range as cell-free transmission (Fig.1A and B). At the highest virus or infected cell input a reduction in luciferase reporter signal is observed due to increased cell death and the resultant loss of infected TZM-bl cells. Input of infected cells was chosen as such that ensuing infection of TZM-bl cells was in the same range as in the free virus infections (virus infectivity corresponding to 5'000- 10'000 relative light units (RLU) per well in absence of inhibitors). To ensure that cell-cell transmission is always probed in the linear range of the assay, a titration of donor cell input, as depicted in Fig. 1A, was included in each individual experiment. Cell-cell virus infections were performed in culture medium containing no DEAE if not otherwise indicated. On day 4 post infection, infected PBMC were washed twice to remove free virions. Cells were then pre-incubated with virus directed inhibitors for 1h before co-culturing with TZM-bl target cells ($1x10^4$ per well). To assess activity of target cell directed agents, TZM-bl cells were pre-incubated with inhibitors before co-culturing with infected PBMC. 48 hours after infection cells were lysed and luciferase reporter gene production measured upon addition of firefly luciferase substrate (Promega, Madison Wisconsin, USA). Inhibitor and antibody concentrations causing 50% reduction in viral infectivity (50% inhibitory concentration, IC50) were calculated by fitting pooled data from three to four independent experiments to sigmoid dose response curves (variable slope) using GraphPad Prism. If 50% inhibition was not achieved at the highest drug concentration a

greater-than value was recorded.

Only R5 viruses which we determined to depend upon DEAE-Dextran to efficiently infect TZM-bl cells as free virus inoculum were used in the PBMC^{HIV+}/TZM-bl assay (Fig. S2). For these isolates no infection was detectable over a wide range of virus input in the absence of DEAE-Dextran. Although this dependence can be overcome at very high virus concentrations, infectivity remained 1-2 orders of magnitude lower. Levels of virus and infected cell input that mediate efficient cell-cell transmission but restrict free virus infectivity were then employed in the assays. R5 and X4 using virus strains which efficiently infect TZM-bl cells in the absence of cationic compounds cannot be used in this assay. While DEAE-Dextran also improves infectivity of these viruses (Fig. S2), residual infectivity in the absence of the polycation is too high and impedes precise discrimination of cell-free and cell-cell infection in the DEAE dependent PBMC^{HIV+}/TZM-bl infection system.

Assessment of free virus and cell-cell transmission using envelope pseudotyped virus particles

We utilized the DEAE dependent TZM-bl infection assay system also to assess single-round virus infection during cell-cell transmission using envelope pseudotyped viruses. The pseudotype backbone used in these experiments (pNLgfp-AM) does not encode for luciferase, which allowed discrimination between donor and target cell infection, as only in the TZM-bl target cells luciferase production will be induced upon infection. Free virus infection in presence of DEAE was performed as described with minor modifications [36]. Cell-free virus input was chosen to yield virus infectivity corresponding to 5'000-10'000 relative light units (RLU) per 96 well in absence of inhibitors. Virus and inhibitors were preincubated for 1h at 37°C in 96 well plates, then TZM-bl (10^4 per well) were added. To assess the neutralization activity of mAbs and inhibitors during cell-cell transmission of pseudovirus, 293-T cells (10^4 per well) were seeded in 96-well plates, and 24h later transfected with 24µg of the pseudovirus backbone pNLgfp-AM and 8µg of the JR-FL env per plate (0.33 µg per well) - using polyetheylenimine (PEI, linear 25 kDa, Polysciences) as transfection agent. Twenty-four hours post transfection virus producing 293-T cells were washed twice with DMEM (10% FCS, P/S) and pre-incubated with virus directed inhibitors (1h at 37°C). Then TZM-bl cells (10^4 per well) were added. No DEAE Dextran was present in the cell-cell transmission setting. After 48 hours of co-culture, infection of the TZM-bl cells was monitored by quantifying the production of the reporter luciferase and the 50% inhibitory concentration (IC50) of the respective drugs was assessed.

Assessment of free virus and cell-cell transmission using rhTRIM5α restricted A3.01-CCR5 cells

The neutralization activity of mAbs and inhibitors was additionally evaluated on cells expressing rhesus (rh) TRIM5α as they have been shown to restrict preferentially free virus transmission (Figure S3B and [51]). A3.01-CCR5 were transduced with human or rhTRIM5α or mock treated and used as target cells in T-T cell transmission experiments and were co-cultivated with infected A3.01-CCR5HIV+. To study free virus infection the same set of target cells were infected with cell-free replication competent virus. TRIM5α expression was monitored by the expression of bi-cistronic expressed GFP. HIV infection of cells was detected by intracellular p24 staining by FACS using the BD Cytofix/Cytoperm Fixation and Permeabilization Kit (BD Biosciences) and mAb KC57-RD1 (anti-HIV-1 p24-Gag, Beckman Coulter), following the manufacturers' instructions. To assess the influence of rhTRIM5α on cell-cell transmission, A3.01-CCR5^{huTRIM5α}, A3.01-CCR5^{rhTRIM5α} or A3.01-CCR5^{mock} cells and either co-cultured with infected donor cells (A3.01-CCR5^{HIV+}) or cell-free virus 6 days post transduction. The same virus stocks were used for free virus infections and to infect donor cells. Infection was monitored after 2-7 days of culture by measuring Gag protein expression in the target cell population. To monitor influence of the transmission mode on entry inhibition cell-cell transmission was assessed by determining efficacy of inhibition using A3.01-CCR5^{rhTRIM5α} and infected donor cells (A3.01-CCR5^{HIV+}). This was compared to inhibition of free virus infection of mock treated A3.01-CCR5 cells. Virus input for both transmission modes was adjusted to yield a comparable output of approximately 10% Gag positive A3.01-CCR5 cells in absence of inhibitors. Inhibitor concentrations which yield maximum inhibition of cell-free virus infection were determined for all compound/virus pairings and probed at these doses in the cell-cell transmission setting. Virus directed inhibitors were preincubated with cell-free replication competent virus or infected A3.01-CCR5 cells (50'000 per 96 well) for 1h at 37°C. A3.01-CCR5^{rhTRIM5α} target cells were added (50'000 per well) and infection allowed to spread for 2-7 days depending on the growth kinetics of the respective isolates. Infectivity was assessed by intracellular p24 staining. % inhibition = 100 – 100 / [% infected cells in uninhibited sample] * [% infected cells in sample x]. As control, infection with cell-free and cell-associated virus was performed using transwell chambers (12-well 0.4 µm polyester-membrane dishes (Corning Life Sciences, Corning, NY) and virus inocula (cell-free or cell- associated) added to the transwell insert. Uninfected human or rhesus TRIM5α transduced A3.01-CCR5 were seeded as target cells in the bottom chamber.

Assessment of free virus and cell-cell transmission using rhTRIM5α restricted PBMC

The $PBMC^{HIV+}$/$PBMC^{rhTRIM5α}$ transmission assays were performed essentially as described for A3.01-CCR5 cells. To assess cell-cell transmission using rhTRIM5α, stimulated, CD8 depleted PBMC were transduced with rhTRIM5α one day after isolation, sorted 4 days post-transduction and co-cultivated with infected PBMC one day after sorting. In parallel cells were mock treated and cell-free inhibition was monitored utilizing the same virus stocks. Infection was monitored after 3 days of culture by measuring Gag protein expression in the target cell population.

Virus attachment assay

Vpr-GFP or Gag-iGFP labeled virion attachment to target cells was studied in presence or absence of entry inhibitors. GFP labeled viruses were preincubated with virus-directed antibodies or inhibitors for 1h at 37°C, then added to wells of a 96-well round-bottom plates containing target cells (PBMC (100'000 cells/well); A3.01-CCR5, A2.01, HeLa, and TZM-bl: (50'000 cells/well)) in a total volume of 100µl. Attachment of virus to target cells was synchronized by spinoculation (2h at 1200g) at 23°C [43]. This low temperature allows efficient attachment of virions to target cells and receptor engagement but impedes virus-cell fusion [123]. Following spinoculation, unbound virus was removed by washing cells twice in FACS buffer (PBS, 2% FBS, 0.1% azide). Cells were then fixed in 1.5% paraformaldehyde (PFA) and GFP positive cells indicative of virus attachment quantified by flow cytometry on a CyAn ADP instrument (Beckman Coulter). Data analysis was performed with FlowJo software (Treestar). The endogenous green fluorescence of mock treated cells (no virus) was determined and the mean fluorescence intensity (MFI) of virus treated samples corrected for this value. 100% attachment (0% inhibition, medium control) was determined in cells treated with virus in absence of inhibitors. The inhibition achieved by the various inhibitors and neutralizing antibodies was expressed relative to this value. % inhibition of attachment = 100 – 100 / [MFI medium control] * [MFI inhibitor x].

Virus fusion assay

We employed a virion-based fusion assay, which detects the enzymatic activity of virion co-packaged β-lactamase post fusion, to assess virus entry essentially as described previously [60, 61]. Virus entry is thereby measured as the extent of cleavage of a cytosolic, fluorogenic substrate by virion co-packaged β-lactamase (BlaM). The latter is achieved by incorporation of chimeric BlaM-

vpr into viral particles which is delivered to the target cells cytosol upon successful entry [61]. To test the pre-attachment inhibitory potency of mAbs and inhibitors, BlaM-Vpr containing viruses were preincubated with nAbs or inhibitors for 1h at 37 °C. Target cells (PBMC/100'000 cells/well or A3.01-CCR5/ 50'000 cells/well) were added and virus attachment initiated by spinoculation (2h, 1'200g, 23°C). Cells were then immediately washed with CO2-independent medium (Gibco) to remove unbound virus and inhibitors. In parallel, to test post attachment activity of entry inhibitors, the inhibitors or nAbs were added after spinoculation (after excess unbound virus had been washed off) and were incubated with the virion bearing cells for 1h at 23 °C. To initiate virus–cell fusion, samples from both the pre and post attachment conditions, were incubated for 3h at 37 °C. The cells were then washed once in medium and loaded with the fluorogenic β-lactamase substrate CCF2/AM (Invitrogen) and incubated for 1h at room temperature following the manufacturer's instructions. Cells were then washed twice in developing medium (CO2-independent medium (Gibco), 2.5 mM probenecid (Sigma), 10% FBS) and incubated overnight at room temperature to allow the β-lactamase to cleave CCF2/AM. Following a wash step with PBS, cells were stained with anti-CD4-APC (Caltag), washed again and fixed in 3% paraformaldehyde. CD4 positive cells were accounted for by flow cytometry and cell populations containing uncleaved CCF2/AM (520nm, no virus fusion) and cleaved CCF2/AM (447nm; virus fusion) determined. Inhibition of fusion was determined by reduction in cell numbers positive for cleaved CCF2/AM (447nm, 450/50 filter) fluorescence and calculated as % inhibition = 100 – 100 / [% infected cells in medium control] * [% infected cells in inhibitor x treated sample].

Virus entry assay based of luciferase reporter gene assay

To analyze post-attachment activity of mAbs and inhibitors, we studied infection of A3.01-CCR5 cells by Env-pseudotyped luciferase reporter viruses. To test pre-attachment activity, inhibitors and mAbs were pre-incubated for 1h at 37°C, and then spinoculated onto A3.01-CCR5 cells as described above. Unbound virus and inhibitors were washed off immediately after spinoculation. To test post-attachment activity, virus was first spinoculated onto A3.01-CCR5 cells, residual virus washed off and then inhibitors incubated with the virus bearing cells for 1h at 23°C. Both pre- and post-attachment cultures were then cultivated for 48h at 37°C before infection was monitored by determining luciferase production as described above.

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Figure 1

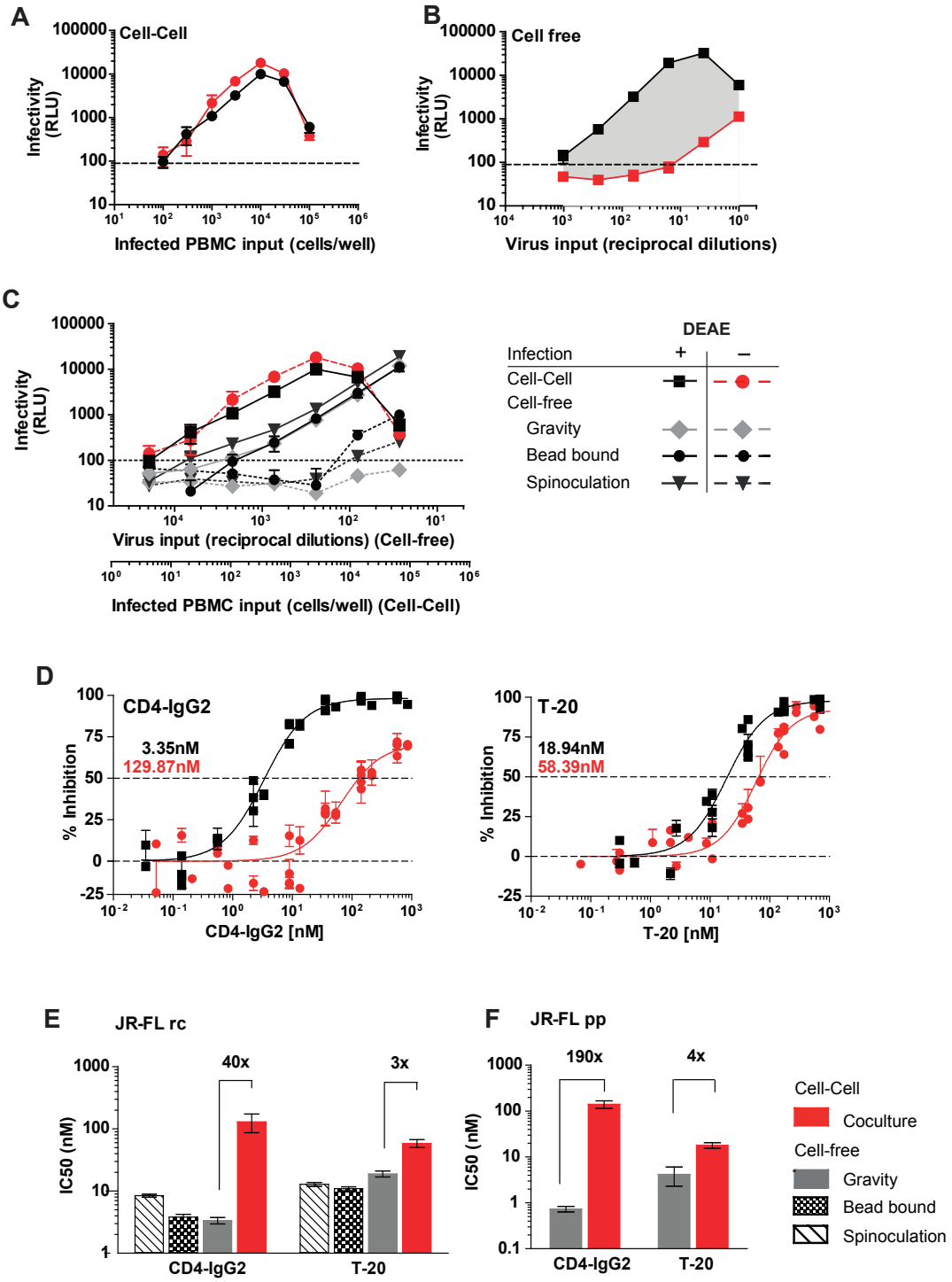
A
Cell-Cell
Infectivity (RLU)
Infected PBMC input (cells/well)
B
Cell free
Infectivity (RLU)
Virus input (reciprocal dilutions)
C
Infectivity (RLU)
Virus input (reciprocal dilutions) (Cell-free)
Infected PBMC input (cells/well) (Cell-Cell)
DEAE
Infection
+
–
Cell-Cell
Cell-free
Gravity
Bead bound
Spinoculation
D
CD4-IgG2
3.35nM
129.87nM
% Inhibition
CD4-IgG2 [nM]
T-20
18.94nM
58.39nM
% Inhibition
T-20 [nM]
E
JR-FL rc
IC50 (nM)
40x
3x
CD4-IgG2
T-20
F
JR-FL pp
IC50 (nM)
190x
4x
CD4-IgG2
T-20
Cell-Cell
Coculture
Cell-free
Gravity
Bead bound
Spinoculation

Figure 1: Mode of virus transmission differentially steers susceptibility to entry inhibition.

(A) DEAE-Dextran is not required for effective cell-cell transmission of HIV-1JR-FL to TZM-bl cells. Serial dilutions of JR-FL infected PBMC were incubated with TZM-bl cells in presence (black circles) or absence (red circles) of 10µg/ml DEAE-Dextran. Infectivity was measured by enzymatic activity of the luciferase reporter (relative light units (RLU)). Each infected cell input was probed in triplicate. Error bars represent SD (standard deviation). One of four independent experiments is shown.

(B) Omission of DEAE-Dextran as media supplement abolishes cell-free JR-FL infection of TZM-bl cells. Serial dilutions of cell-free JR-FL virus were used to infect the luciferase reporter cell line TZM-bl in presence (black squares) or absence (red squares) of 10µg/ml DEAE-Dextran. Infectivity was measured by induction of the luciferase reporter (relative light units (RLU)). Each virus dilution was probed in quadruplicates. Bars represent SD . One of four independent experiments is shown.

(C) Cell-cell transmission but not enforced contact between virus and target cell overcomes entry restriction. The infectivity of cell-free virus without enforced attachment to TZM-bl target cells (gravity sedimentation), or upon spinoculation, magnetic bead virus adsorption and during cell-cell transmission was assessed in presence (solid lines) or absence (dotted lines) of 10µg/ml DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each virus dilution was probed in duplicates. Bars represent SD. One of three independent experiments is shown

(D) Inhibitory profiles of CD4-IgG2 and T-20 during cell-cell and cell-free transmission. TZM-bl target cells were either cocultivated with JR-FL infected PBMC (red circles, no DEAE) or cell-free virus (black squares, with 10µg/ml DEAE) in the presence of increasing doses of CD4-IgG2 (left panel) or T-20 (right panel). Infection was determined by measuring luciferase production after 48h and recorded as RLU. Red and black values denote IC50 (nM) of during cell-cell and cell-free transmission, respectively. Data points represent means of duplicates from three independent inhibition experiments. Bars represent SEM. Lines depict fitted dose response curves.

(E) Decreased CD4-IgG2 sensitivity during cell-cell transmission is due to an inherent feature of cell-cell transmission. TZM-bl target cells were mixed with replication competent infected JR-FLrc PBMC in the presence of CD4-IgG2 or T-20 (red bars) in medium lacking DEAE Dextran. Cell-free JR-FLrc was either spinoculated (hatched bars), adsorbed by magnetic beads (checkered bars) or added without enforced adsorption (grey bars) onto TZM-bl target cells in medium containing DEAE Dextran in the presence of the inhibitor. Fold increases in IC50 of cell-cell compared to cell-free infection are indicated on top of the respective bars. Bars depict means of three independent experiments in duplicates .Lines denote SD. Inhibition of cell-cell transmission by CD4-IgG2 and T-20 (red bars) was significantly less efficient than blocking of cell-free virus (grey bars) infection (Student t-test, $p<0.0001$ in both cases).

(F) Single round infection is highly resistant to CD4-IgG2 inhibition during cell-cell transmission. TZM-bl target cells (no DEAE) were co-cultivated with JR-FL pseudovirus transfected 293-T cells in the presence of CD4-IgG2 or T-20. Cell-free JR-FLpp-lucAM was added to the TZM-bl (with 10µg/ml DEAE) in the presence of both inhibitors. Fold increases in IC50 of cell-cell compared to cell-free infection are indicated on top of the respective bars. Bars depict means of three independent experiments performed in duplicates. Lines denote SD. Inhibition of cell-cell transmission by CD4-IgG2 and T-20 (red bars) was significantly less efficient than blocking of cell-free virus (grey bars) infection (Student t-test, $p<0.0001$ in both cases).

Figure 2

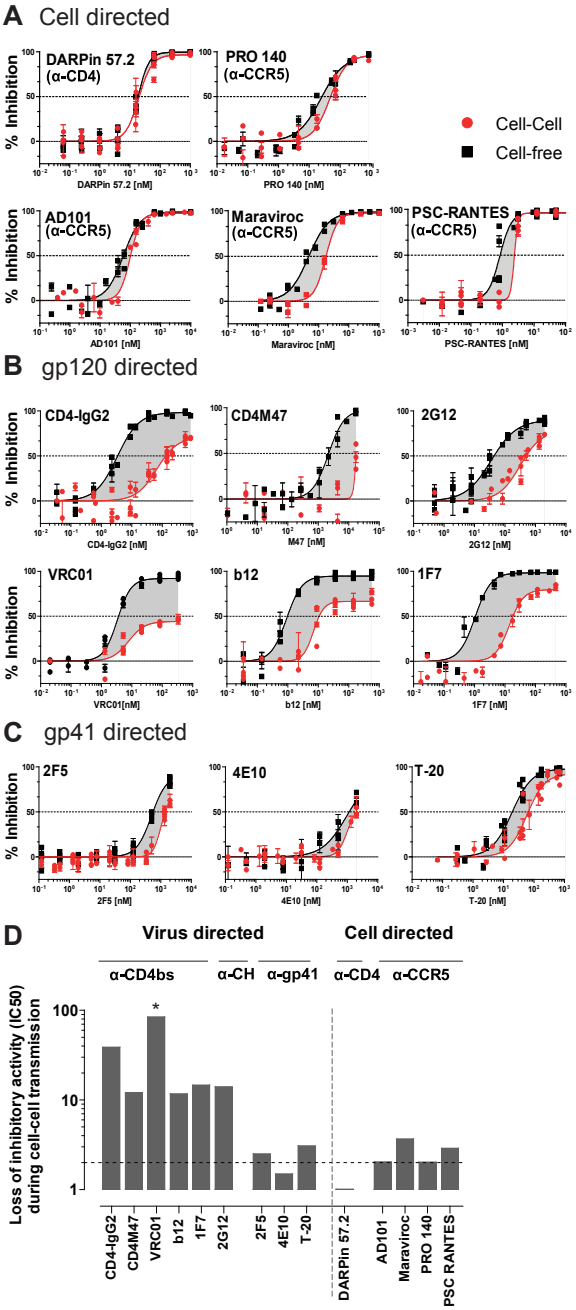

Figure 2: **Markedly decreased sensitivity of HIV entry to gp120 directed inhibitors during cell-cell transmission.**
(A-C) TZM-bl target cells were either infected with cell-free JR-FL^{rc} (black squares, with DEAE) or cocultivated with JR-FL^{rc} infected PBMC (red circles; no DEAE) and inhibition by cell directed (A), gp120 directed (B) and gp41 directed (C) antibodies and inhibitors studied. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Lines depict fitted results derived from three to five independent experiments in which each sample condition was performed in duplicates. Error bars depict SEM. Dotted lines indicate 50% inhibition levels.
(D) Loss of inhibitory activity during cell-cell transmission. Loss of inhibitory activity during cell-cell transmission compared to cell-free transmission is depicted as fold difference of IC50 values determined from data depicted in Fig 2A-C. A star (*) denotes where the respective inhibitor did not reach a 50 % inhibition level at the highest concentration used. The highest concentration probed was used in these cases as minimum estimate to derive the fold differences in IC50 values.

Figure 3

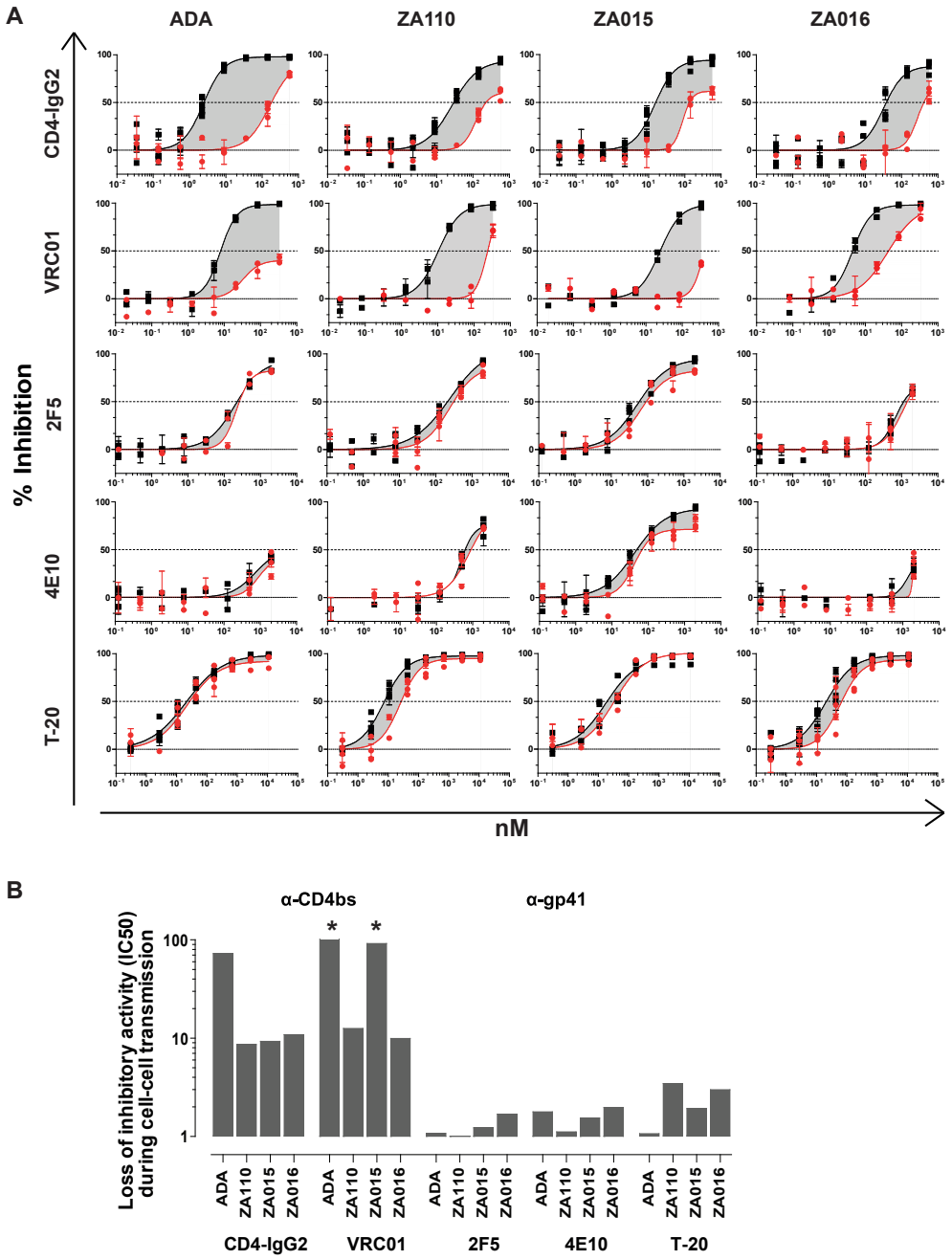

Figure 3: CD4bs directed inhibitors loose while gp41 directed agents maintain activity during cell-cell transmission across divergent HIV-1 isolates.

(A) TZM-bl target cells were either infected with cell-free, replication competent viruses (black squares, with DEAE) or cocultivated with infected PBMC (red circles; no DEAE) and inhibition by the indicated antibodies and inhibitors studied. Virus isolates used (ADA, ZA110, ZA015 and ZA016) are indicated on top of the respective columns, inhibitors on the left of the respective rows. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Lines depict fitted results derived from two to three independent experiments in which each sample condition was probed in duplicates. Error bars depict SEM. Dotted lines indicate 50% inhibition levels.

(B) Loss of inhibitory activity during cell-cell transmission. Loss of inhibitory activity during cell-cell transmission compared to cell-free transmission is depicted as fold difference of IC50 values determined from data depicted in Fig 3A. A star (*) denotes where the respective inhibitor did not reach a 50 % inhibition level at the highest concentration used. The highest concentration probed was used in these cases as minimum estimate to derive the fold differences in IC50 values.

Figure 4

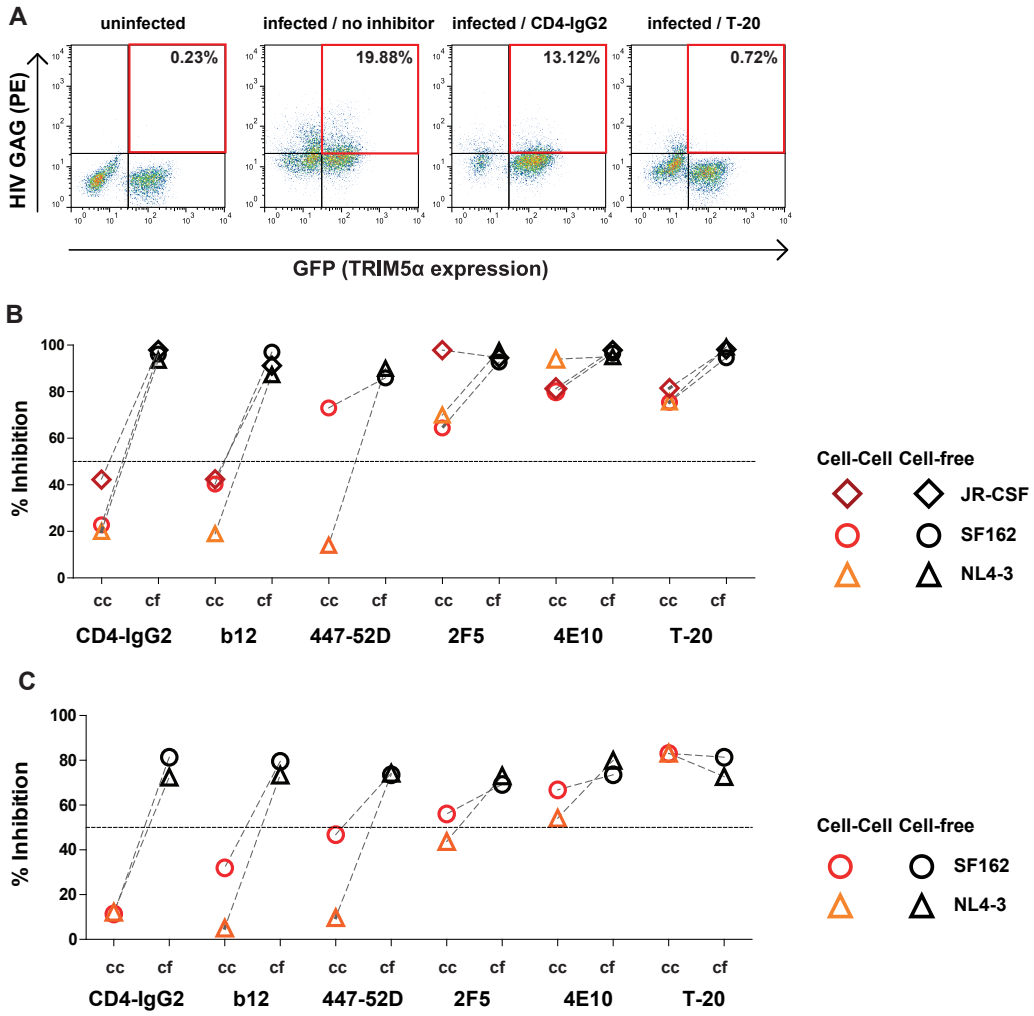

Figure 4: Efficient inhibition of T cell to T cell transmission by gp41 directed inhibitors.

(A) Inhibition of T-cell to T-cell transmission. A3.01-CCR5 infected with JR-CSF or uninfected controls were co-cultured with A3.01-CCR5$^{rhTRIM5\alpha}$ target cells (GFP positive) in the presence of the indicated inhibitors or medium alone. Infection of target cells was assessed by intracellular Gag staining by flow cytometry. Percentages of rhTRIM5α expressing, HIV infected cells are indicated. One representative of two independent experiments is shown.

(B) Comparison of cell-free and cell-cell inhibition in rhTRIM5α restricted A3.01-CCR5 cells. Inhibition of cell-cell (cc, red and orange symbols) and cell-free (cf, black symbols) transmission of virus isolates JR-CSF, SF162 and NL4-3 by inhibitors (CD4-IgG2, VRC01, b12 and 447-52D: 50 µg/ml, 2F5, 4E10: 100µg/ml, T-20: 5µg/ml) was studied. To probe cell-cell transmission infected A3.01-CCR5 were cocultured with A3.01-CCR5$^{rhTRIM5\alpha}$ target cells. To study free virus transmission cell-free virus preparations were used to infect non-restricted A3.01-CCR5 cells. Infection of target cells was assessed by intracellular Gag staining by flow cytometry as described in A). Infection achieved in absence of inhibitor was set to 100% and inhibitor activity expressed in relation to this value. Data depicted are means of two to seven independent experiments.

(C) Comparison of cell-free and cell-cell inhibition in rhTRIM5α restricted PBMC. Inhibition of cell-cell (cc, red and orange symbols) and cell-free (cf, black symbols) transmission of virus isolates SF162 and NL4-3 by inhibitors (CD4-IgG2, VRC01, b12 and 447-52D: 50 µg/ml, 2F5, 4E10: 100µg/ml, T-20: 5µg/ml) was studied. To probe cell-cell transmission infected PBMC were cocultured with PBMC$^{rhTRIM5\alpha}$ target cells. To study free virus transmission cell-free virus preparations were used to infect non-restricted PBMC cells. Infection of target cells was assessed by intracellular Gag staining by flow cytometry as described in A). Infection achieved in absence of inhibitor was set to 100% and inhibitor activity expressed in relation to this value. Data depicted are means of two independent experiments in duplicates.

Figure 5

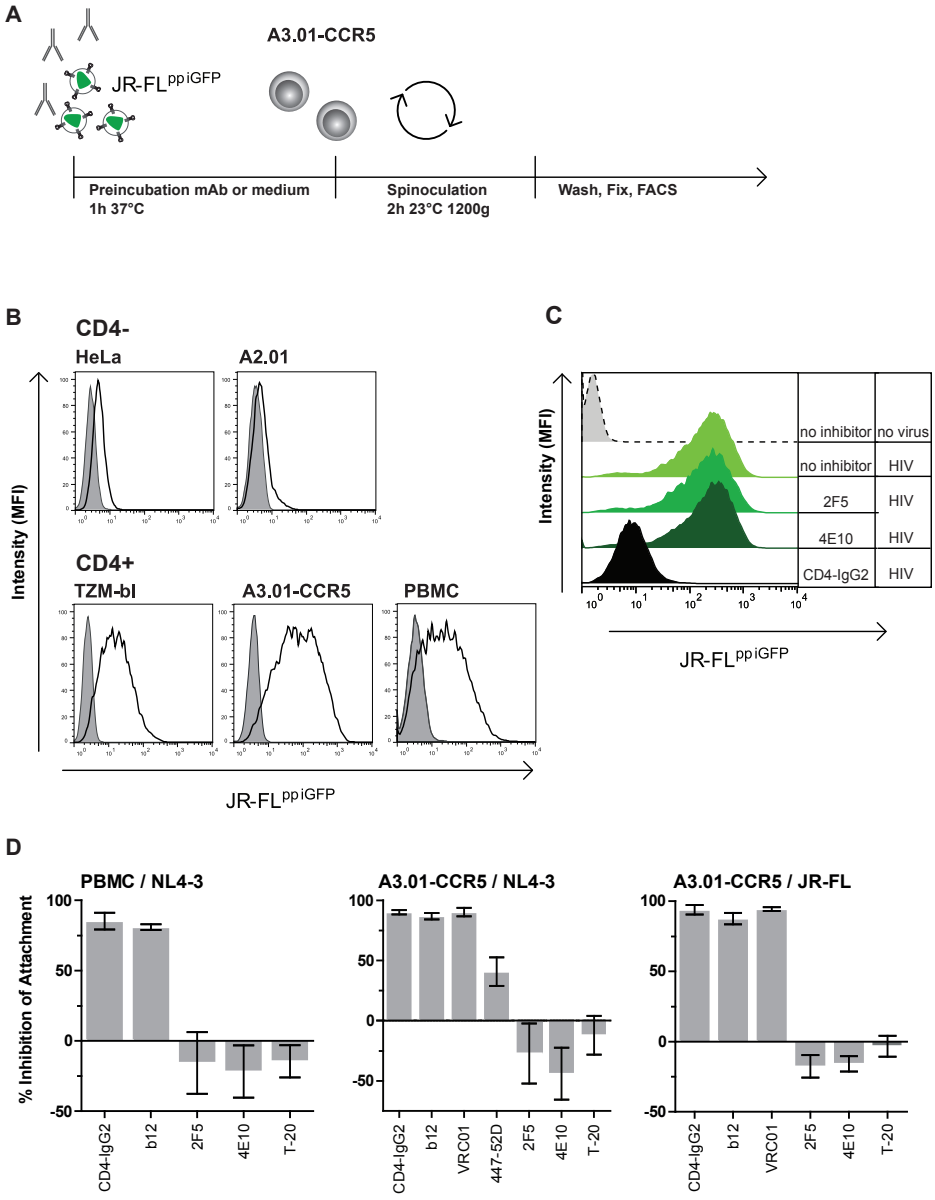

Figure 5: Attachment of virus is blocked by preventing gp120-CD4 interaction.

(A) Schematic illustration of the experimental set up used to analyze virus attachment.

(B) Attachment of virus is driven by binding to CD4. Attachment of HIV to CD4 negative (HeLa, A2.01) and related CD4 positive cells (TZM-bl, A3.01-CCR5) as well as stimulated, CD8 depleted PBMC was studied using GFP-labeled virus (JR-FL^{ppiGFP}). The gray-shaded areas represent the fluorescent signal obtained by flow cytometric analysis of the respective cell line in the absence of HIV. The black lines indicate fluorescence intensity of bound JR-FL^{ppiGFP}. (C) Influence of entry inhibitors on HIV attachment. Activity of 2F5, 4E10 and CD4-IgG2 to block attachment of GFP-labeled virus (JR-FL^{ppiGFP}) to A3.01-CCR5 cells is shown (Inhibitor concentration listed in Table S2). Histograms of one representative of three independently performed experiments are shown.

(D) Inhibition of HIV attachment by CD4bs and gp41 directed agents. Attachment (MFI of GFP signal) achieved in absence of inhibitor was set to 100% and inhibitor activity expressed in relation to this value. Data depicted are means of three independent experiments, error bars denote SEM. Left panel: Attachment of Vpr-GFP labeled TN8 virus (NL4-3 envelope) to PBMC. Middle panel: Attachment of GFP-labeled virus (JR-FL^{ppiGFP}) to A3.01-CCR5. Right panel: Attachment of GFP-labeled virus (NL4-3^{ppiGFP}) to A3.01-CCR5 cells. (Inhibitor concentration listed in Table S2)

Figure 6

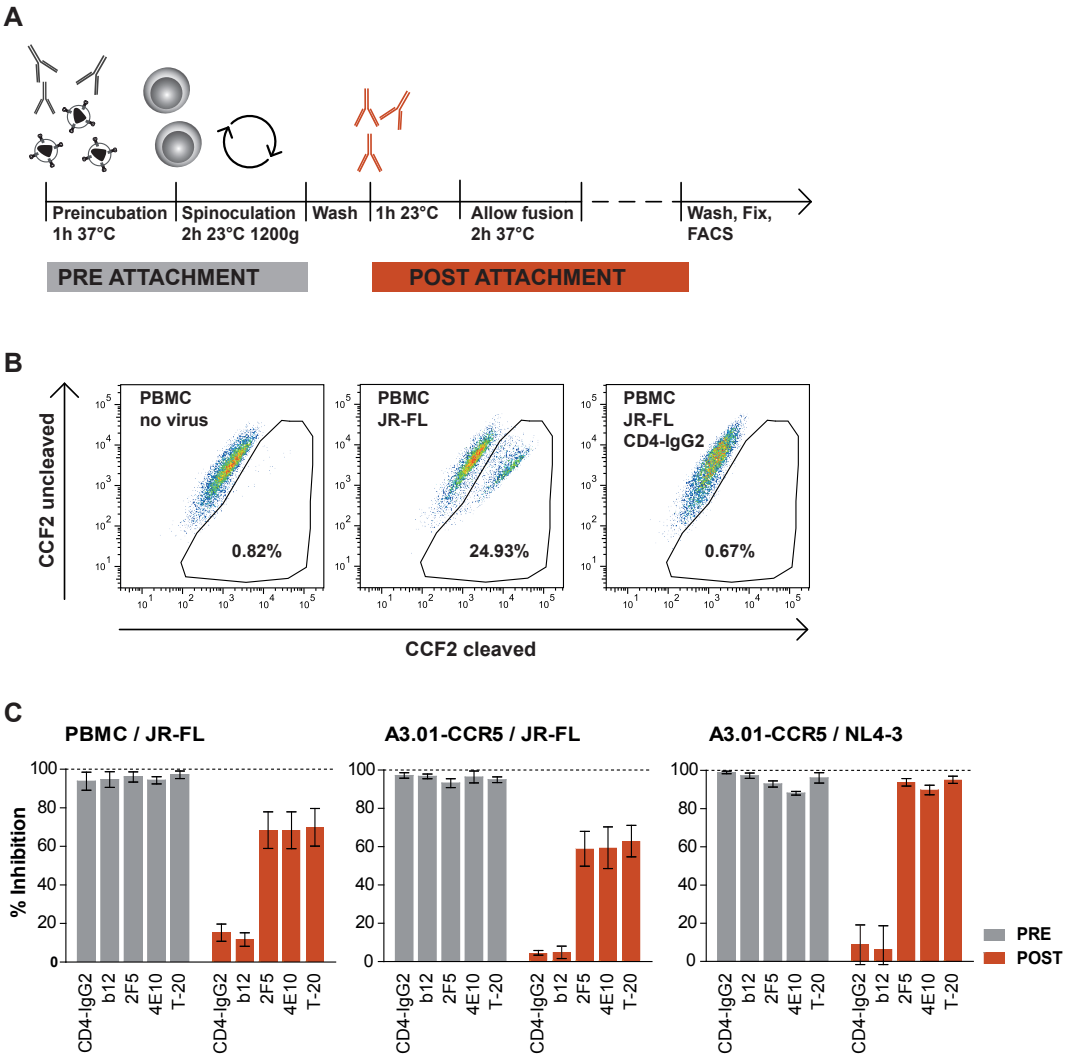

Figure 6: Post attachment activity of entry inhibitors.

(A) Schematic illustration of the entry assay using BlaM-Vpr labeled virions.

(B) JR-FL$^{pp\text{-}BlaM}$ entry into PBMC was studied in presence and absence of CD4-IgG2 (50µg/ml). One of three individual experiments is shown. Fluorescence of uncleaved CCF2/AM was recorded at 520nm, β-lactamase cleaved CCF2/AM denoting HIV entry at 447nm.

(C) Inhibition of virus entry. Fusion of JR-FL$^{pp\text{-}BlaM}$ and NL4-3$^{rc\text{-}BlaM}$ with PBMC or A3.01-CCR5 cells was monitored in presence and absence of the indicated entry inhibitors (Inhibitor concentration listed in Table S2). Grey and orange bars correspond to pre- and post-attachment conditions respectively as depicted in (A). Data shown are means of three independent experiments, error bars denote SEM.

Figure 7

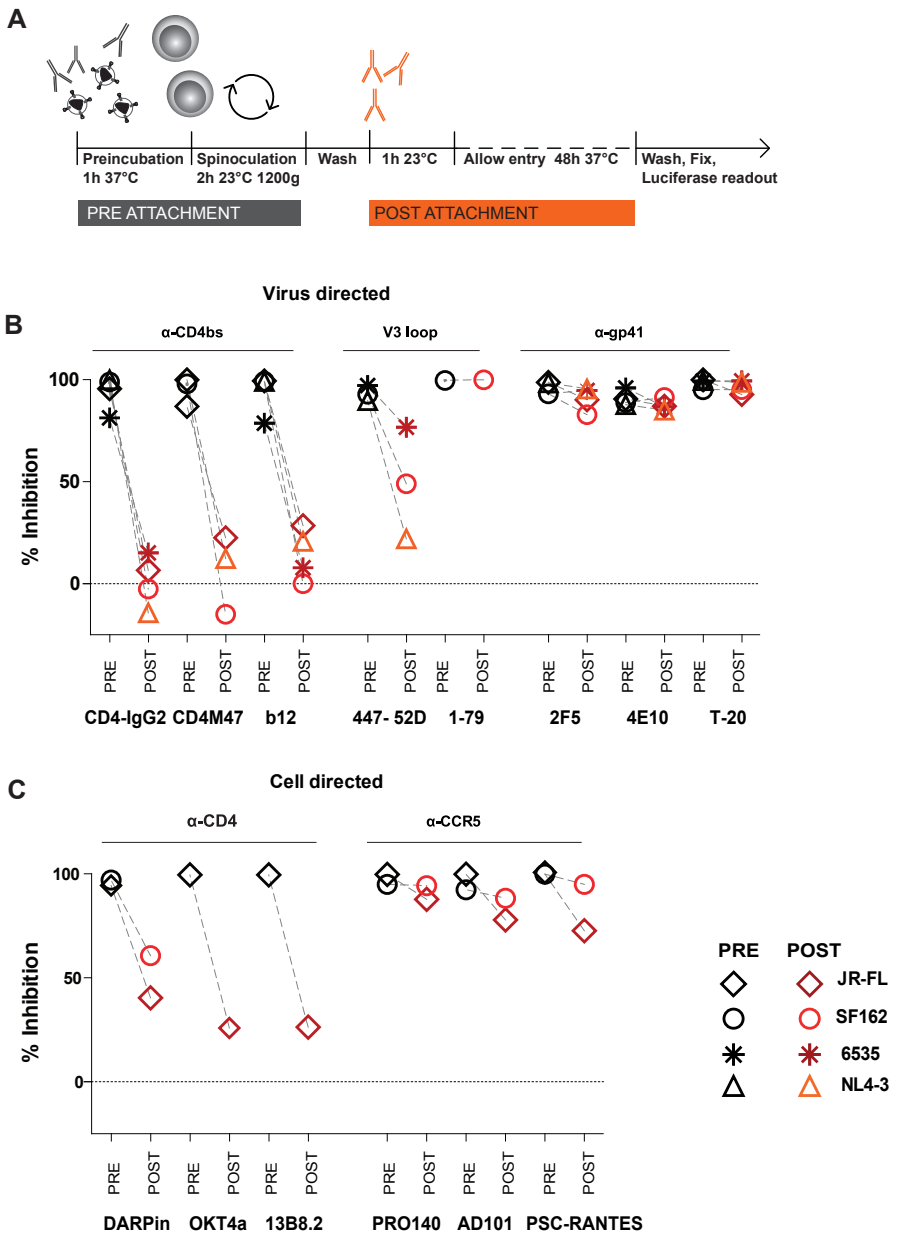

Figure 7: Gp41 specific inhibitors have broad post-attachment activity.
(A) Schematic illustration of the luciferase reporter assay utilized to assess post attachment activity of inhibitors.
(B) Post-attachment activity of virus directed inhibitors. A3.01-CCR5 cells were infected and treated with inhibitors before or after virus attachment as indicated in (A). (Inhibitor concentration listed in Table S2). Infection of env-pseudotyped, luciferase reporter viruses JR-FL$^{pp\text{-}lucAM}$ (diamonds), SF162$^{pp\text{-}lucAM}$ (circle), 6535$^{pp\text{-}lucAM}$ (star), NL4-3$^{pp\text{-}lucAM}$ (triangle) was determined after 48h of culture by measuring luciferase production (recorded as RLU). Data depict means of pre attachment (black symbols) and post attachment activity (red and orange symbols) as % inhibition compared to untreated control. Means of three to six independent experiments are shown.
(C) Post-attachment activity of cell directed inhibitors. A3.01-CCR5 cells were infected and treated with inhibitors before or after virus attachment as indicated in (A) (Inhibitor concentration listed in Table S2). Infection of env-pseudotyped, luciferase reporter viruses JR-FL$^{pp\text{-}lucAM}$ (diamonds) and SF162$^{pp\text{-}lucAM}$ (circle)was determined after 48h of culture by measuring luciferase production (recorded as RLU). Data depict means of pre attachment (black symbols) and post attachment activity (red and orange symbols) as % inhibition compared to untreated control. Means of two to four independent experiments are shown.

Figure S1

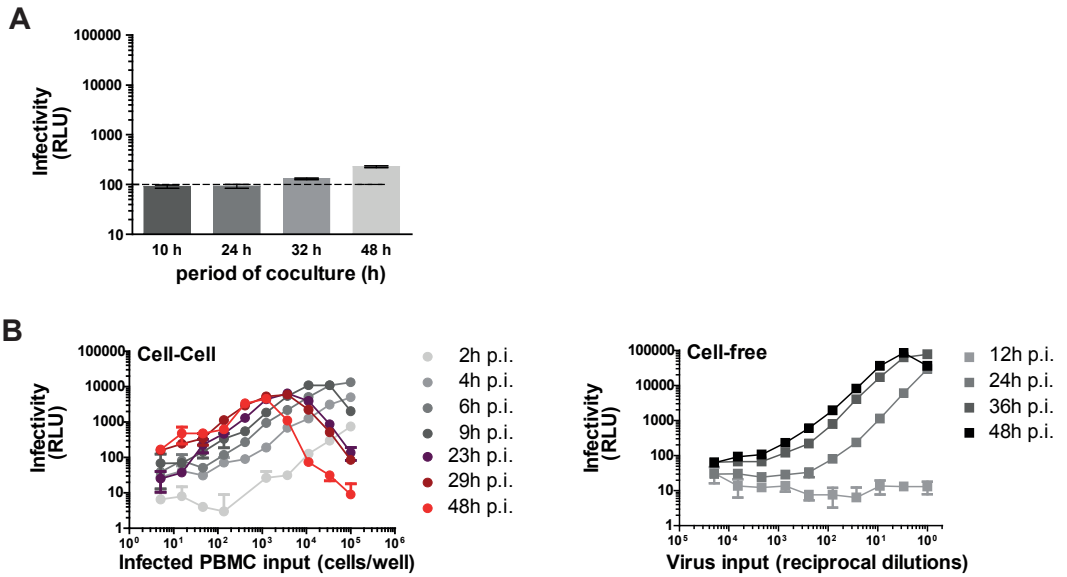

Figure S1: **Dependence of R5 viruses on DEAE-Dextran during cell-free transmission**

(A) Free virus released from infected donor cells during cell-cell transmission has no impact on assessment of cell-cell transmission. JR-FL infected PBMCs were co-cultured with HeLa cells (CD4 and CCR5 negative) to mimic co-culture condition in the PBMC^{HIV+}/TZM-bl infection system without allowing cell-cell transmission to occur. Supernatant was harvested at the indicated time points, transferred onto TZM-bl cells and assessed for infectivity in absence of DEAE-Dextran. During the 48h co-culture period only minute amounts of virus are released from the infected PBMC which fail to infect in the absence of DEAE-Dextran. Thus, at the chosen infected cell input, virus transmission in the PBMC^{HIV+}/TZM-bl infection system in absence of DEAE-Dextran occurred almost exclusively through cell-cell transmission. Data are derived from one of two independent experiments. Means and SEM of triplicate samples are shown.

(B) Cell-cell transmission is more rapid than cell-free transmission. Cell-cell transmission of JR-FL from infected PBMC to TZM-bl in absence of DEAE Dextran (left panel) and cell-free JR-FL infection of TZM-bl in presence of 10µg/ml DEAE-Dextran (right panel) was monitored at the indicated time points by determining luciferase reporter production (RLU). Data points are means of triplicate measurements. Bars represent SEM.

Figure S2

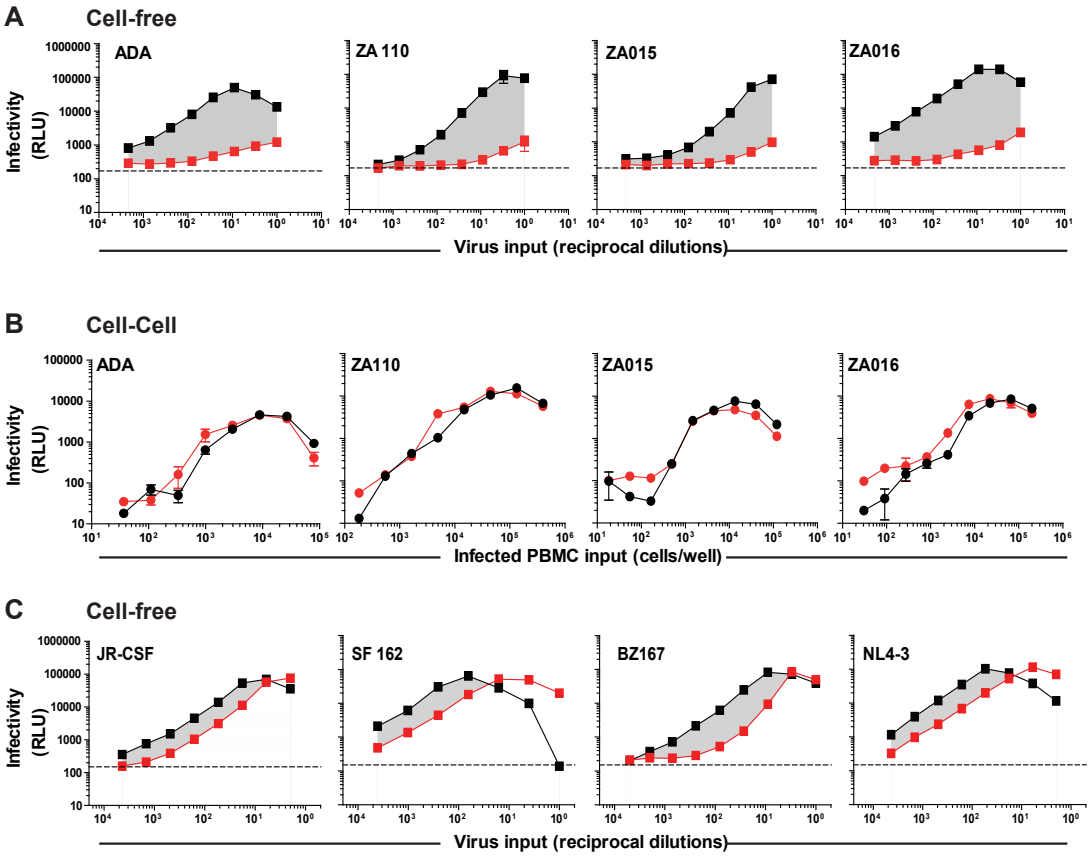

Figure S2: R5 viruses differ in their DEAE-Dextran dependence during cell-free transmission

(A) DEAE-Dextran dependent cell-free infection of TZM-bl cells by R5 viruses TZM-bl cells were infected with serial dilutions of cell-free R5 virus isolates (ADA, ZA110, ZA015 and ZA016) in presence (black squares) or absence (red squares) of 10µg/ml DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each virus dilution was probed in quadruplicates. Bars represent SEM. One of two independent experiments is shown.

(B) Absence of DEAE-Dextran as media supplement has no effect on cell-cell transmission of HIV-1 to TZM-bl cells. Serial dilutions of PBMC infected with different R5 isolates (ADA, ZA110, ZA015 and ZA016) were incubated with TZM-bl cells in presence (black circles) or absence (red circles) of DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each infected cell input was probed in triplicate. Error bars represent SEM. One of two independent experiments is shown.

(C) DEAE-Dextran independent cell-free infection of TZM-bl cells by certain R5 and X4 using viruses. TZM-bl cells were infected with serial dilutions of cell-free R5 virus isolates JR-CSF and SF162, the R5X4 virus BZ167 and the X4 strain NL4-3 in presence (black squares) or absence (red squares) of DEAE-Dextran. Infection was determined by measuring luciferase production after 48h (recorded as RLU). Each virus dilution was probed in quadruplicates. Bars represent SEM. One of two independent experiments is shown.

Figure S3

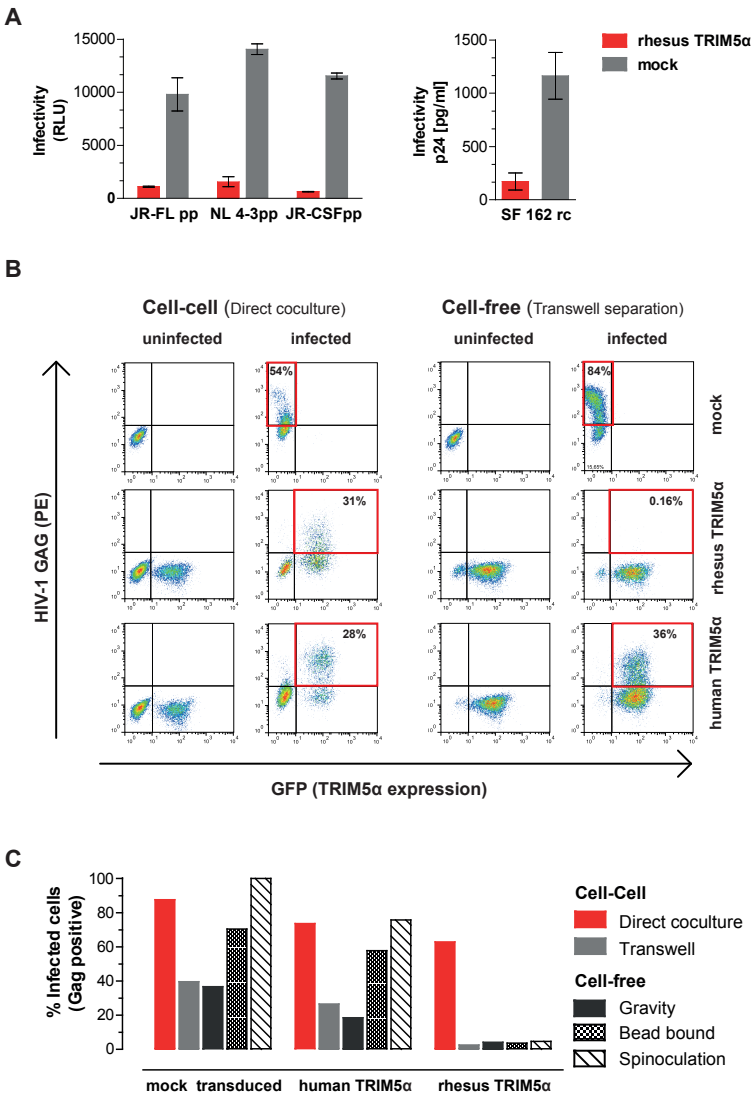

Figure S3: Rhesus TRIM5α restriction allows precise dissection of cell-free and cell-cell transmission of HIV-1.
(A) Rhesus TRIM5α transduced cells are highly resistant to cell-free single round and multiple round infection. Infection of rhesusTRIM5α or mock transduced A3.01-CCR5 cells with the indicated env-pseudotyped, luciferase reporter viruses (left panel) or replication competent SF162 isolate (right panel). Infection of the reporter virus was determined by measuring luciferase production after 48h (recorded as RLU/ml). Infection of SF162 was monitored by determining p24 antigen production. Both cell-free infection with single round, env pseudotyped virus and replication competent virus isolates proved to be almost completely restricted in rhTRIM5α transduced A3.01-CCR5 cells. One of two independent experiments for each virus isolate is shown. Error bars represent SEM.(B) Cell-cell transmission overcomes rhTRIM5α mediated restriction of HIV-1. Uninfected or SF162-infected A3.01-CCR5 cells (donors) were co-cultivated with the indicated A3.01-CCR5 target cells (mock treated (no gfp), rhTRIM5α (gfp positive), huTRIM5α (gfp positive)) either in direct coculture (left panel or separated by transwells (right panel). Infection was assessed by intracellular HIV-1 Gag staining after 6 days of coculture. Data show one representative out of three independent experiments.
(C) Cell-cell transmission but not enforced contact between virus and target cell overcomes rhTRIM5α mediated entry restriction. Comparison of the infectivity of cell-free SF162 infection of i) spinoculated, ii) magnetic bead bound virus and iii) virus added without enforced adsorption with cell-cell transmission (direct cocultivation and transwell). Infection of mock treated, rhTRIM5α and huTRIM5α A3.01-CCR5 target cells was investigated. One representative out of three independent experiments is depicted. To allow comparison, data are normalized to infection levels obtained by spinoculating cell-free SF162 onto mock transduced cells.

Figure S4

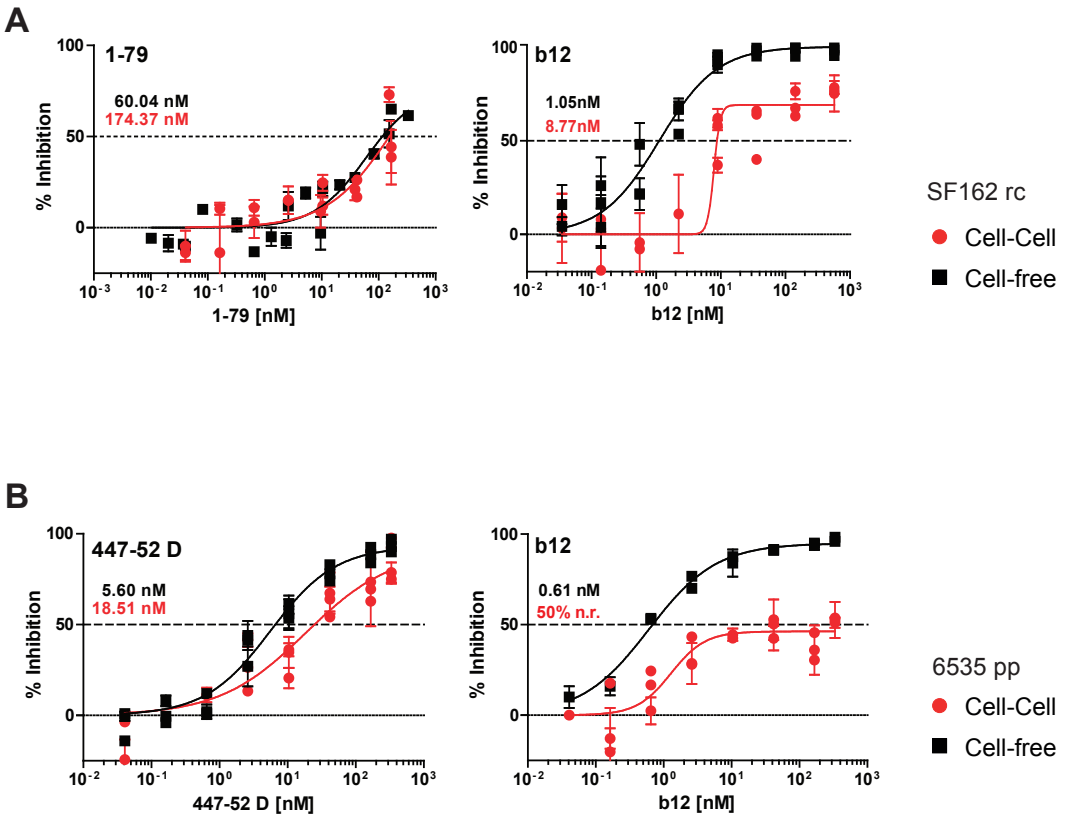

Figure S4: Efficient inhibition of Cell-Cell transmission by V3 directed antibodies

(A) V3 directed antibody 1-79 efficiently inhibits cell-cell transmission of replication competent SF162. Activity of V3 loop mAb 1-79 and CD4bs directed mAb b12 to inhibit cell-cell transmission was studied by co-cultivating rhTRIM5α transduced TZM-bl with SF162rc infected PBMC (red circles; no DEAE in infection media). Inhibition of free virus transmission of SF162rc was monitored in parallel on TZM-bl target cells in absence of rhTRIM5α (black squares; 10µg/ml DEAE in infection media). Infection was determined by measuring luciferase production after 48h (recorded as RLU). Lines depict fitted results derived from three independent experiments in which each sample condition was performed in duplicates. Error bars depict SEM. (B) Single round infection by 6535 is sensitive to 447-52D inhibition during cell-cell transmission. Activity of V3 loop mAb 447-52D and CD4bs directed b12 to inhibit cell-cell transmission was studied by co-cultivating rhTRIM5α transduced TZM-bl with 6535 pseudovirus transfected 293-T cells (red circles; no DEAE in infection media). Inhibition of free virus transmission of cell-free $6535^{pp\text{-}lucAM}$ was monitored in parallel on TZM-bl target cells in absence of rhTRIM5α (black squares; 10µg/ml DEAE in infection media). Infection was determined by measuring luciferase production after 48h (recorded as RLU). Lines depict fitted results derived from three independent experiments in which each sample condition was performed in duplicates. Error bars depict SEM.

Supplementary Table 1: Origin and specificity of mAbs and inhibitors

Inhibitor	Drug class	Target	Epitope (HxB2) / Binding site	MW	Reference	Source
CD4-IgG2	tetrameric CD4 molecule	gp120	CD4bs	175 kDa	Allaway et al.1995 *AIDS Res Hum Retroviruses.11(5):533-9.*	W.Olson, Progenics
CD4M47	miniprotein mimetic	gp120	CD4bs	3 kDa	Stricher et al. 2008 *J Mol Biol, 2008. 382(2): 510-24*	J.Robinson
VRC01	Antibody	gp120	CD4bs	150 kDa	Wu, Yang et al. 2010 *Science.329(5993):856-61*	J.Mascola
b12	Antibody	gp120	CD4bs	150 kDa	Barbas et al. 1992 *PNAS.89(19):9339-43*	D.Burton
1F7	Antibody	gp120	CD4bs	150 kDa	Buchacher et al.1994 *AIDS Res Hum Retroviruses.10(4):359-69* Kunert et al.1998 *AIDS Res Hum Retroviruses. 14(13):1115-28*	H.Katinger, Polymun
2G12	Antibody	gp120	carbohydrate	150 kDa	Trkola et al.1996 *J Virol.70(2):1100-8*	H.Katinger, Polymun
447-52D	Antibody	gp120	V3 (312-315)	150 kDa	Gorny et al.1992 *J Virol.66(12):7538-42*	S. Zolla-Pazner, NIH ARRRP
1-79	Antibody	gp121	V3 (312-315)	151 kDa	Scheid et al. 2009. *Nature* . 458:636–640.	M. Nussenzweig
2F5	Antibody	gp41	MPER (662-667)	150 kDa	Muster et al.1993 *J Virol.67(11):6642-7*	H.Katinger, Polymun
4E10	Antibody	gp41	MPER (671-676)	150 kDa	Stiegler et al. 2001 *AIDS Res Hum Retroviruses. 10;17(18):1757-65* Zwick et al. 2001 *J Virol.75(22):10892-905*	H.Katinger, Polymun
T-20 (Fuzeon)	Peptide, fusion inhibitor	gp41	Fusion peptide	4.5 kDa	Wild et al.1993 *AIDS Res Hum Retroviruses. 9(11):1051-3*	Purchased from Roche Pharmaceuticals
DARPin 57.2	Designed Ankyrin Repeat Protein	CD4	CD4 domain 1	18 kDa	Schweizer, Rusert et al. 2008 *PLoS Pathog. 4(7):e1000109*	A.Trkola
OKT4a	Antibody	CD4	CD4 domain 1	150 kDa	Nicholson et al.1986 *The Journal of Immunology. 137(1): 323-329.*	Orthomune
13B8.2	Antibody	CD4	CD4 domain 1	150 kDa	Benkirane et al.1993. *EMBO J. 242(1): 233-237.*	Q. Sattentau
AD101 (SCH-350581)	Small molecule inhibitor	CCR5	Tansmembrane region of CCR5	0.5 kDa	Strizki, Xu et al. 2001 *PNAS. 98(22):12718-23*	J.Stritzki, Schering Plough
Maraviroc	Small molecule inhibitor	CCR5	Transmembrane region of CCR5	0.5 kDa	Dorr, Westby et al. 2005 *Antimicrob Agents Chemother. 49(11):4721-32*	Purchased from Pfizer
PRO 140	Antibody	CCR5	Nt and ECL2 domains	150 kDa	Olson, Rabut et al. 1999 *J Virol.73(5):4145-55*	W.Olson, Progenics
PSC-RANTES	Chemokine derivative	CCR5	ECL2	8 kDa	Hartley, Gaertner et al. 2004 *PNAS.101(47)16460-16465*	O.Hartley

Table S2. Antibody and inhibitor concentrations

	Figure 4	Figure 5 and 6	Figure 7
CD4-IgG2	50 ug/ml	50 ug/ml	5 ug/ml
CD4M47	-	-	5 ug/ml
b12	50 ug/ml	50 ug/ml	10 ug/ml
VRC01	-	50 ug/ml	-
2F5	100 ug/ml	100 ug/ml	100 ug/ml
4E10	100 ug/ml	100 ug/ml	100 ug/ml
T-20	5 ug/ml	10 ug/ml	5 ug/ml
DARPin 57.2	-	-	1 uM
OKT4a	-	-	10 ug/ml
13B8.2	-	-	50 ug/ml
PRO 140	-	-	100 ug/ml
AD101	-	-	10 uM
PSC-RANTES	-	-	1 uM

5. Summary

Increasing knowledge over the last years has consolidated the evidence that the humoral immune system plays an important role in containing HIV-1 infection. A few broadly neutralizing antibodies have been isolated and proven to successfully protect against HIV-1 infection upon passive immunization ((Trkola, Kuster et al. 2005; Hessell, Poignard et al. 2009; Mascola and Montefiori 2010) reviewed in (Huber, Olson et al. 2008). However, so far vaccine development has failed to induce sufficient antibody responses to mediate sterilizing immunity (Stamatatos, Morris et al. 2009; Mascola and Montefiori 2010). Thus, the precise mechanisms of action of neutralizing antibodies in vivo still need to be unraveled.

The concept of HIV cell-cell transmission across virological synapses (VS) is now well established, and the rapid progress in the field has provided a wealth of information on the structure and function of the VS ((Sato, Orenstein et al. 1992; Dimitrov, Willey et al. 1993; Carr, Hocking et al. 1999; Chen, Hubner et al. 2007; Sourisseau, Sol-Foulon et al. 2007; Mazurov, Ilinskaya et al. 2010) reviewed in (Sattentau 2010)) and has emphasized its potential in vivo relevance (Jung, Maier et al. 2002; Pope and Haase 2003; Hladik, Sakchalathorn et al. 2007).

However, conflicting results have been published concerning the susceptibility of the VS to entry inhibitors and neutralizing antibodies (Ganesh, Leung et al. 2004; Keele, Van Heuverswyn et al. 2006; Chen, Hubner et al. 2007; van Montfort, Nabatov et al. 2007; Massanella, Puigdomenech et al. 2009; Martin, Welsch et al. 2010). The wide range of different assay systems applied to assess cell-cell transmission increase intricacy and confuse the comparison of these results. The main drawbacks of the existing experimental approaches is the lack of direct comparison of cell-cell and cell-free transmission in the same settings (Jolly, Kashefi et al. 2004; Chen, Hubner et al. 2007; van Montfort, Nabatov et al. 2007; Hubner, McNerney et al. 2009; Massanella, Puigdomenech et al. 2009), and the missing quantification of inhibitory activity (Jolly, Kashefi et al. 2004; Keele, Van Heuverswyn et al. 2006; van Montfort, Nabatov et al. 2007).

We first developed specific experimental strategies to compare cell-cell and cell-free transmission in the same setting and to unambiguously discriminate between both transmission modes. Two different approaches to dissect cell-cell transmission from cell-free virus infection were utilized. In a first set up, we utilized the fact that infection of the TZM-bl reporter cell line with specific R5 viruses requires the polycation DEAE-Dextran in the infection medium. Therefore, omission of this polycation reduces cell-free infection. Although a preceding screen for DEAE dependency is inevitable, this set up allows assessing the activity of neutralizing antibodies and entry inhibitors during cell-cell transmission in a standardized and high-through put manner. In a second approach we exploited the capacity of cell-cell transmission to overcome restriction of cell-free infection by rhesus TRIM5α (Richardson, Carroll et al. 2008). RhTRIM5α was introduced in primary PBMC, in a T-cell line as well in the reporter cell line TZM-bl, which allowed adapting the cell-cell

transmission assay to different virus strains independent of their coreceptor usage or rounds of replication. Importantly, the clear dissection of cell-cell and cell-free virus transmission in all assay systems was crucial to measure the potency of inhibitors and neutralizing antibodies in inhibiting cell-cell transmission.

Employing the developed assay systems, we were able to assess the activity of a broad panel of entry inhibitors and neutralizing antibodies during cell-cell transmission. Even though the assay systems had a completely different mode of inhibiting free virus restriction, our analysis revealed identical results in both assay systems. The cell directed inhibitors such as CCR5 and CD4 targeting compounds were equally active during both transmission modes. However, the virus directed inhibitors showed a dichotomous pattern. While gp120 directed inhibitors and neutralizing antibodies, in particular CD4bs directed agents, showed markedly decreased potency in blocking cell-cell transmission, the probed gp41 directed inhibitors, the fusion inhibitor T-20 and the MPER antibodies 2F5 and 4E10, demonstrated identical or only marginally reduced potency. This dichotomy of the virus directed inhibitors was also found in the capacity of these inhibitors and neutralizing antibodies to block the viral entry process. Whereas gp120 directed inhibitors and neutralizing antibodies were able to efficiently inhibit attachment, they lost remarkably potency when added post-attachment. In contrast, MPER mAbs were not able to inhibit attachment, but blocked fusion, both when added during or after attachment.

Thus, we hypothesize that the different time frame of target accessibility defines the potency in cell-cell transmission inhibition. CD4 and CCR5 receptors can immediately be blocked by inhibitors as they are accessible on target cells. In contrast, viral envelope becomes only accessible once it is expressed on the cell surface of the infected cell. Gp120 directed inhibitors only have a narrow time slot to act before virological synapse formation is initiated, mirrored in the significant loss of activity during cell-cell transmission. In contrast, the activity of the MPER NAbs and T-20 is maintained due to their potency to react preferentially in a cellular context blocking the entry steps following HIV envelope engagement by CD4. The processes required for the transition from receptor engagement to fusion have been shown to be slow enough for these agents to act. Altogether, our findings show that cell directed inhibitors and virus directed inhibitors which interfere with later steps in the entry process block efficiently HIV-1 cell-cell transmission.

Additional differences between the two transmission modes have to be considered in order to discuss the possible reasons for the measured differences in cell-cell inhibition. Whether HIV-1 transfer across the virological synapse leads to fusion at the host cell membrane or is mediated by endocytosis still remains to be resolved (reviewed in (Permanyer, Ballana et al. 2010)). The accumulation of HIV Gag in the endosomal compartment of a target cell after cell-cell transmission has been reported by several groups (Blanco, Bosch et al. 2004; Chen, Hubner et al. 2007; Hubner, McNerney et al. 2009) but it is still controversially discussed whether it is a cell type de-

pendent observation or holds true as a paradigm for all virological synapse mediated infections (Permanyer, Ballana et al. 2010; Sattentau 2010). However, if endocytosis was a preferred entry pathway after cell-cell transmission, it seems unlikely that it would offer protection against the humoral immune response given the high potency of some neutralizing antibodies to block cell-cell transmission (our results and (van Montfort, Nabatov et al. 2007; Massanella, Puigdomenech et al. 2009; Martin, Welsch et al. 2010).

It has been observed that virus particle morphogenesis and transfer to a target cell occur almost simultaneously. Due to its rapid spread through virological synapses, immature and therefore fusion inefficient particles might get transmitted to a target cell and fuse from within the endosomal compartment as soon as maturation is complete (Dale, McNerney et al. 2011). Aiken et al. reported that exposure of the gp41 epitopes recognized by the MPER specific mAb 4E10 was increased on the surface of immature particles, and suggested that MPER specific bNAbs act by trapping gp41 in a conformational intermediate during particle maturation (Joyner, Willis et al. 2011). Thus, one could hypothesize that the increased ability of MPER-directed antibodies to inhibit cell-cell transmission is also based on their capacity to bind immature virions and block subsequent maturation and entry. Hence to define and characterize the subsequent entry steps and virion maturation after virological synapse mediated infection remains a challenging task and further experiments are needed to fully investigate cell-cell transmission and it's in vivo implications.

It has been extensively described that HIV escapes antibody responses rapidly (Richman, Wrin et al. 2003; Wei, Decker et al. 2003; Frost, Wrin et al. 2005; Trkola, Kuster et al. 2005; Manrique, Rusert et al. 2007). Even the most potent and broadly active antibodies characterized in recent years (Binley, Wrin et al. 2004; Walker, Simek et al. 2010; Wu, Yang et al. 2010; Zhou, Georgiev et al. 2010; Scheid, Mouquet et al. 2011; Tomaras, Binley et al. 2011; Walker, Huber et al. 2011) have been isolated from chronically infected individuals who fail to control viremia. Our observations might offer an explanation why these antibodies are so potent in vitro and still fail to suppress viremia. We were able to show that the blocking activity of CD4bs antibodies is largely directed towards free virus, but inefficient against cell-cell transmission. Therefore, despite their extraordinary breadth and potency they allow virus replication and spread to occur via cell-cell transmission. Considering the possible correlation between control of viremia and potency in inhibition of cell-cell transmission, it seems prudent to incorporate cell-cell transmission studies in future vaccine assessment to determine whether or not activity in blocking cell-cell transmission is a correlate of protection.

It is generally agreed that neutralizing antibody responses will be a essential component of an effective HIV vaccine (Hope 2011; Walker, Ahmed et al. 2011)). However, whether vaccine elicited neutralizing antibodies will need to block only cell-free virus or whether protection will also require restriction of both cell-free and cell-cell transmission with equal efficacy, still needs to be defined.

Despite the growing knowledge of the virus itself and its interaction with the host, we still lack -after 30 years of HIV discovery (Barre-Sinoussi, Chermann et al. 1983)- fundamental knowledge regarding the nature of the immune response needed for protection. Natural immunity itself is far from effective, both humoral and cellular immune response are induced but do not lead to clearance or protecting against HIV superinfection (Altfeld, Allen et al. 2002). Thus, to investigate and understand immunity against HIV is a major goal in HIV-1 research and will ultimately facilitate exploiting it for prevention and therapy.

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9. Publication list

Abela IA, Berlinger L, Schanz M, Reynell L, Günthard HF, Rusert P, Trkola A. *Cell-cell transmission enables HIV-1 to partially evade inhibition by potent CD4bs directed antibodies. PloS Pathogens 2012, in Press*

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